Journal of
DAIRY SCIENCE

Vol. 41 September, 1958 Number 9

CONTENTS

People and Events 3

Student Chapter News 19

Research Papers:

Bovine Mastitis: A Review. W. N. Plastridge 1141

Factors Influencing Growth and Toxin Production in Cheese Inoculated with Spores of* Clostridium Botulinum *Types A and B. I. Studies with Surface-Ripened Cheese Type I. R. O. Wagenaar and G. M. Dack . . 1182

Factors Influencing Growth and Toxin Production in Cheese Inoculated with Spores of* Clostridium Botulinum *Types A and B. II. Studies with Surface-Ripened Cheese Type II. R. O. Wagenaar and G. M. Dack . . 1191

Factors Influencing Growth and Toxin Production in Cheese Inoculated with Spores of* Clostridium Botulinum *Types A and B. III. Studies with Surface-Ripened Cheese Type III. R. O.Wagenaar and G. M. Dack . . 1196

***Identity of Esters Produced in Milk Cultures of* Pseudomonas Fragi.** J. N. Pereira and M. E. Morgan 1201

Isolation and Identification of Acidic and Neutral Carbonyl Compounds in Different Varieties of Cheese. E. W. Bassett and W. J. Harper . . 1206

Observations on the Reduction of 2,3,5-Triphenyltetrazolium Chloride by Homofermentative Lactic Acid Bacteria. B. J. Liska, H. E. Calbert, and S. G. Knight 1218

Calcium Excretion in Human Subjects at Two Levels of Milk Intake. Johnnie H. Watts and D. C. W. Graham 1224

High Roughage System for Raising Calves Based on the Early Development of Rumen Function. VIII. Effect of Rumen Inoculations and Chlortetracycline on Performance of Calves Fed High Roughage Pellets. J. W. Hibbs and H. R. Conrad 1230

High Roughage System for Raising Calves Based on Early Development of Rumen Function. IX. Effect of Rumen Inoculations and Chlortetracycline on Rumen Function of Calves Fed High Roughage Pellets. H. R. Conrad, J. W. Hibbs, and Norma Frank 1248

Influence of Anise Oils on the Palatability of Calf Starters. W. J. Miller, J. L. Carmon, and H. L. Dalton 1262

Sodium, Potassium, Calcium, and Chloride Distribution in Bovine Semen. R. G. Cragle, G. W. Salisbury, and N. L. VanDemark 1267

Distribution of Bulk and Trace Minerals in Bull Reproductive Tract Fluids and Semen. R. G. Cragle, G. W. Salisbury, and J. H. Muntz . . 1273

Variations in Quality of Liquid and Frozen Semen at Various Temperatures. D. F. Weseli, T. M. Ludwick, E. A. Hess, and F. Ely 1278

Corn versus Oat Silages as a Roughage for Dairy Cattle. C. A. Lassiter, C. F. Huffman, S. T. Dexter, and C. W. Duncan 1282

(Continued on inside front cover)

CONTENTS

(Continued from front cover)

Technical Notes:

Composition of Casein in the Smallest Casein-Containing Particles of Milk. T. F. Ford and Jose Martinez-Mateo 1286

Adaptability of the Fisher-Stern Ultracentrifuge in Studying the Sedimentability of Milk Proteins. H. K. Wilson and E. O. Herreid . . . 1287

The High-Melting Glyceride Fraction from Milk Fat. Stuart Patton and Philip G. Keeney 1288

Effect of Potassium Orotate and Methionine Alone and with Various Antibacterial Agents in a High Colostrum Diet for Young Calves J. M. Wing 1290

Fertility of Bovine Semen Extended at 28–30° C. in Egg Yolk–Citrate with or Without Glycerin. J. W. Snyder and E. L. Farmer 1291

Our Industry Today:

A Fully Mechanized Method for the Manufacture of Cheddar Cheese. W. W. McCadam and H. Leber 1293

Our Association:

Report of Committee on Butter, A.D.S.A. 1300

Abstracts of Literature A57

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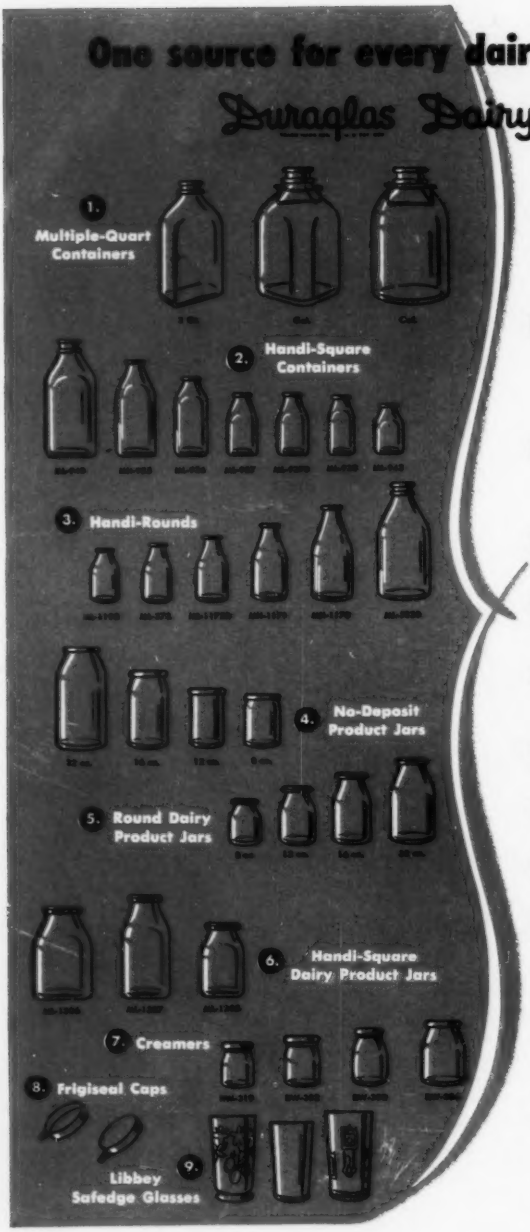

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PEOPLE AND EVENTS

Letter to the Secretary-Treasurer from the U. S. Undersecretary of Agriculture

Dear Mr. Judkins:

Thank you for your letter of June 27 listing the resolutions which were adopted at the Annual Meeting of the American Dairy Science Association at Raleigh, North Carolina, June 16-19, 1958. We are commenting on each of the resolutions in the order in which they were listed in your letter:

1. The continued interest of the American Dairy Science Association in animal health programs is most encouraging and reflects a sound appreciation of the needs in this regard. Substantial progress is being made in the cooperative State-Federal brucellosis eradication campaign. During the past fiscal year, the States of Connecticut, Pennsylvania, Michigan, Vermont, New Jersey, Utah, New Mexico, Rhode Island, and the Commonwealth of Puerto Rico qualified as Modified-Certified areas. This makes 15 states and Puerto Rico which have achieved this distinction, and others are rapidly approaching the same status. With the $20,000,000 provided by Congress for the brucellosis program during fiscal year 1959, and a substantial amount available from the States, it should be possible to continue the cooperative eradication effort at an effective level. As you know, a considerable amount of work remains to be done in the "certified" states as well as in the others.

Projects are being carried on to develop effective control and eradication procedures for mastitis, vibriosis, leptospirosis, and other such diseases. However, until improved methods and materials are made available through research, concerted efforts to combat these diseases on a nationwide basis must, of necessity, be delayed.

In the case of mastitis, control and eradication are seriously handicapped by the fact that a variety of factors, including management practices, may be either directly or indirectly responsible for the condition. Moreover, the accurate diagnosis of infectious mastitis requires time-consuming laboratoy examinations. The effectiveness of available treatments is intimately related to the identification of organisms involved. Improved diagnostic procedures and more effective treatments are needed.

Although vibriosis appears to be relatively widespread throughout many sections of the country, complete control and eventual eradication would be difficult, if not impossible, to achieve on a nationwide basis with available tools. The blood agglutination test is of limited value and much inferior to the vaginal mucus test. The latter test is a laboratory procedure used mainly for herd diagnosis. The only conclusive identification of vibrio fetus infection depends upon cultural isolation of the organism. This is a detailed operation which does not lend itself to practical field program operations. Studies are in progress along lines designed to develop improved diagnostic and therapeutic procedures relating to vibriosis.

Limited serological testing and field observations have not as yet established the extent of leptospirosis in this country. Control of the disease is retarded by lack of basic knowledge about the condition as it affects a variety of animal species. Although vaccination against leptospirosis has given encouraging results, its real value and limitations have not been established. Here again, additional information must be obtained from research before effective control and eradication procedures can be applied as action programs in the field.

2. We are pleased to advise you that Congress has passed a bill which the President has signed into law, authorizing the continuation of the Special Milk Program through June 30, 1961. The law authorizes expenditure of $75,000,000 in each of those years for operation of the program.

With the invaluable assistance and cooperation of the State agencies, the Department will continue to operate the Special Milk Program in the same manner as it has been operated. Preliminary figures for the 1958 fiscal year show that more than 75,000 schools and child-care institutions participated in the program, serving nearly 2 billion half-pints of milk to children. Expenditures under the program for the 1958 fiscal year are estimated at approximately $67,000,000. Further Special Milk Program expansion is expected this year.

3. There has been considerable activity by the various dairy departments of the Land-Grant Colleges to interest more high-school students in dairying. Relative to the level of pay of men trained in dairy science, this is determined largely by Congress and the pay rates in the various grades. During the past year, two changes have taken place: *(1)* the present session of Congress authorized an increase of 10 per cent in level of pay for scientists, which partially relieves the competition with educational and commercial institutions; (2) this Department has requested the Civil Service Commission's approval to hire at Grade GS-11 rather than GS-9 Ph.D. graduates in the biological and closely related sciences, including those trained in dairy husbandry. These two changes would make a marked improvement in our competitive position in recruitment efforts. The Department will continue to work toward the solution of this problem.

4. The commendation by the Association of the Dairy Cattle Research Branch of the Agricultural Research Service for its efforts in the production record programs for dairy cows is gratifying. The maintenance and dissemination

of current data are essential to an effective breeding program designed to produce better sires. The increased acceptance of sire-proving in recent years has made it necessary for the Department to thoroughly eveluate the procedures and methods of maintaining up-to-date records. Our study shows that this increase in volume of data used in sire-proving can not be kept current by methods presently in use. As a result, we have had under consideration for some time the need to shift the entire process to electronic data equipment. We are determined to make the shift as soon as available resources will permit.

5. Review of the records available in the Agricultural Research Service indicates that various milk-metering devices for pipe-line milkers now available from commercial sources either have been tested or are in process of being tested at several of the state experiment stations. These include experiment stations in Illinois, Maryland, Michigan, Minnesota, North Carolina, Virginia, and Wisconsin.

Since farmers generally look to their state experiment stations for recommendations on this type of equipment, the manufacturers usually cooperate with the experiment stations by making equipment available for testing and evaluation. Information obtained from one state experiment station indicates that manufacturers of milk-metering devices probably have made one or more of these devices available to a considerable number of state experiment stations for testing and research. Tests of limited scope oftentimes are conducted without having a specific project and, thus, may not be known until the results are published. We are quite confident that experiment stations generally are cognizant of the need for accurate, unbiased information on milk-metering devices, and that they will do their part in providing information useful to dairymen.

TRUE D. MORSE
Undersecretary,
Department of Agriculture,
Washington, D. C.
August 7, 1958

Research Specialties Co. News

The establishment of a new Chromatographic Materials Dept. has been announced by Research Specialties Co. This new department provides for the first time a single source of supply for small lots of the great variety of substances and reagents used in laboratory applications of the various chromatographic processes.

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Gessert Joins Upjohn Veterinary Staff

R. A. GESSERT, D.V.M., formerly with U.S. Food and Drug Administration, has joined the staff of The Upjohn Company Veterinary Medical Dept. as one of 14 veterinarians employed by the Kalamazoo pharmaceutical firm. He is a large-animal specialist and has extensive background in the fields of bovine ketosis and nutrition.

A native of Michigan and a graduate of Michigan State Univ., Dr. Gessert did his undergraduate work in the field of dairy husbandry, followed by graduate work in the general field of animal nutrition. He received his D.V.M. degree in 1950.

After four years in the Army, Dr. Gessert was in private practice first, and then worked as a research associate at the Univ. of Maryland, specializing in metabolic diseases of dairy cattle and in rumen nutrition.

From 1955 until his employment by Upjohn, Dr. Gessert was an assistant veterinary medical director for F.D.A. in Washington, D. C., where he processed new drug applications, checked labeling for compliance with the law, and served as a consultant to other F.D.A. officials on veterinary matters.

Dr. Gessert is a member of the American Veterinary Medical Association, the American Association of Veterinary Physiologists and Pharmacologists, and the American Association of Veterinary Nutritionists. In the latter group, he has been particularly active in relaying feed medication information to practicing veterinarians. Dr. Gessert has published many papers on the subjects of bovine ketosis, animal nutrition, and the relationship of F.D.A. to veterinary medicine.

Ohio News

The Dept. of Dairy Technology at Ohio State Univ. has conducted a survey of the Ohio dairy industry to determine its needs and wishes with respect to adult education programs.

Results of the survey showed definite interest in a management program dealing with personnel and research interpretation and application. The department will plan its program in this direction.

Completed Theses

Ph.D. Degree

J. E. LONG—The physical properties and interactions of some minor milk proteins. Ohio State Univ., Columbus.

M.S. Degree

J. H. MARTIN—Factors affecting antibiotic activity in milk. Ohio State Univ., Columbus.

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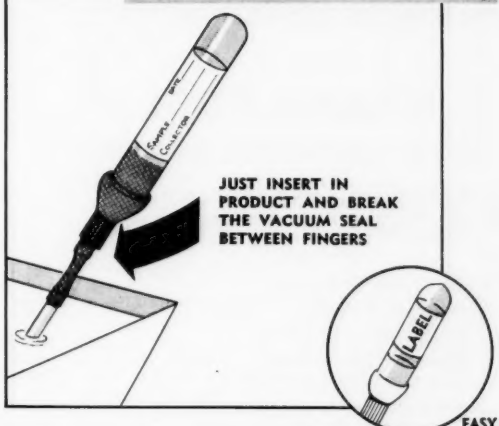

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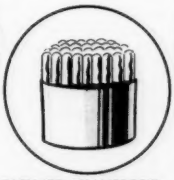

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Illinois News

F. H. DODD, visiting professor in the Dept. of Dairy Science at the Univ. of Illinois for the past year, has returned to his post at the National Institute of Research in Dairying. Prof. Dodd has worked with new milk-handling and milk-metering equipment during his stay at Illinois.

International Congress Postponed

The Fourth International Congress on Animal Reproduction has been postponed from 1960 until 1961. The Congress will be held in 1961, in The Netherlands, so that it may be held simultaneously with the next Congress on Animal Production.

A Message from Your National Membership Committee

Your student affiliate and regular membership drives will be initiated on October 1, 1958. By now, you should have noticed some membership activity in your state through the State Membership Chairman and his Committee. For the benefit of those who may not be familiar with the organization of the membership committee, a diagrammatic sketch of the organization is shown on page 7.

It is noted that a chairman and associate chairman have been designated for each of the divisions and the two regions within the Western Division. The chairman is responsible for the organization and for carrying out of the membership drive for regular members. For the membership program to be most effective, it is necessary for the divisional and regional chairmen to work closely with the state chairmen. The divisional and regional chairmen also have the responsibility for coordinating the student affiliate membership program through the associate chairmen at the divisional, regional, and state levels. The Associate Chairman of the National Membership Committee primarily will be responsible for the student affiliate membership program. He has some good ideas in mind for the coming year which should expedite this program.

Your assistance in helping the committees carry out their assignments in accordance with the schedule will be deeply appreciated by the Membership Committee — J. B. FRYE, JR., W. W. SNYDER, W. S. ARBUCKLE, L. R. DOWD, C. A. ERNSTROM, E. L. THOMAS, H. A. BENDIXEN, F. B. WOLBERG, G. E. STODDARD, P. M. REAVES, J. E. JOHNSTON, J. A. ELLIOTT, GEORGES BELANGER, D. L. GIBSON.

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(11 states)

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(3 states)
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EACH STATE

Chairman and 5 members
1 Associate Chairman (In charge of Student Affiliate Membership)
1 Representing Production
1 Representing Manufacturing
1 Representing Industry (Such as equipment, feed, etc., closely related to the Dairy Industry)
1 At Large

IMPORTANT DATES

Type of member	*Initiation of drive*	*Termination of drive*	*Clean-up campaign*
(1) Student affiliate	Oct. 1, 1958	Dec. 15, 1958	Dec. 15, 1958, to Feb. 1, 1959
(2) Regular	Oct. 1, 1958	Jan. 1, 1959	Jan. 1, 1959, to Feb. 1, 1959

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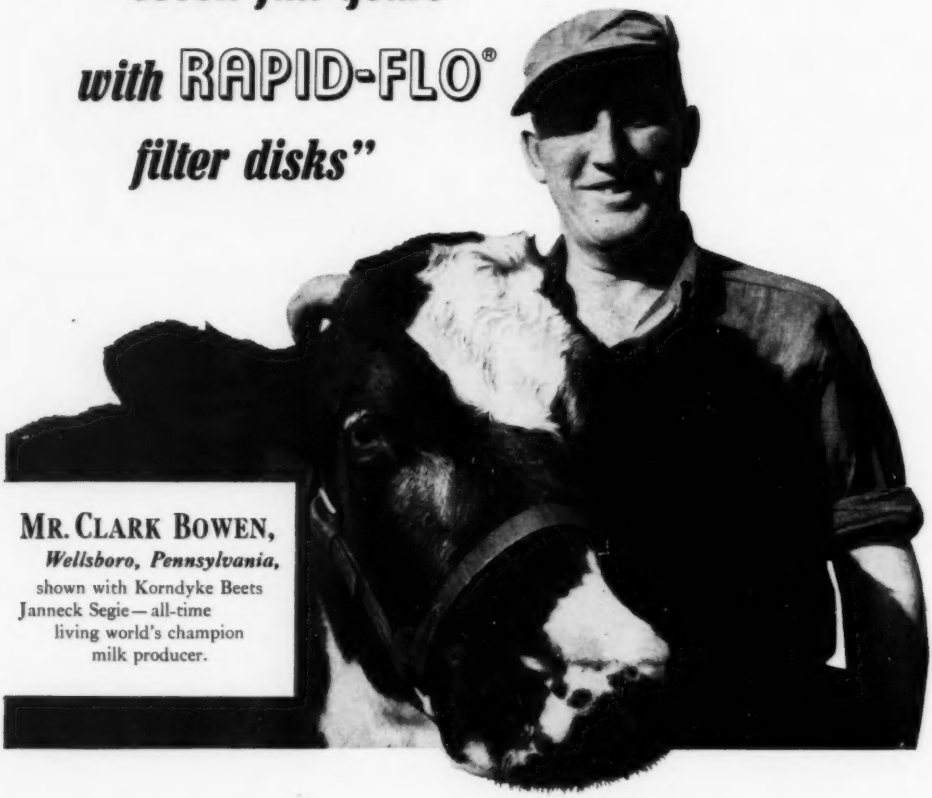

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F. H. Herzer Retires

F. H. HERZER, head of the Dairy Dept. at Mississippi State Univ., retired July 1, 1958, after more than 39 years of service to the University and the Mississippi dairy industry. During his retirement, Mr. Herzer will continue as secretary-treasurer of the Mississippi Dairy Products Association, a post he has held since 1944.

F. H. Herzer

In April, 1958, this distinguished Southern educator was voted the "Faculty Member of the Year" by alumni of Mississippi State Univ., the first time this recognition had been given.

He was also presented the first Milk Industry Foundation "Master Teaching Award in Dairy Manufacturing and Administration" at the 1955 annual A.D.S.A. meeting at Michigan State Univ. This award included an honorarium of $1,000. Earlier that year, he was awarded the citation "For Distinguished Service to the Dairy Industy of the South" by the Southern Division of the A.D.S.A.

Mr. Herzer was educated in the public schools of Marion, O., and received his B.S. degree from Ohio State Univ. in 1914 and his M.S. degree from Iowa State College in 1935.

After graduation from O.S.U., he worked as a short course instructor at Ohio State, became an instructor in dairying at the Univ. of Arkansas, and entered the Naval Aviation Corps during World War I.

In the fall of 1919, he became associate professor in the Dairy Dept. at Mississippi State College and immediately organized the dairy manufacturing curriculum, using the A & M Co-operative Creamery as a laboratory. At the same time, he started the college ice cream department with a tub freezer and ice crusher. He also founded one of the first milk pasteurizing plants in Mississippi which furnished the student-body with bottled, pasteurized milk.

Through the years, ice cream and milk sales funds have paid for the Dairy Industries building and equipment. Since his appointment as acting head of the department in 1946 and as head in 1949, Mr. Herzer has guided the reorganization of the dairy production division, the expenditure of some $350,000 in barns and milking parlor, and an expanded program in teaching and research.

His research activities have been directed toward such subjects as "The Effect of Feeds on the Fat Constants, Flavor, and Texture of Southern Butter;" "The Vitamin-A Potency of Mississippi Butter;" "The Chemical Composition of Mississippi Milk;" "The Factors Affecting the Flavor and Body of Mississippi Milk;"

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1 **Finest ingredients.** Only top-grade cocoa and sucrose are used in Kraft's powder. We harvest our own Irish Moss, extract the stabilizer from it with our own careful process. Because all ingredients must meet our rigid specifications, you are assured uniformity of color, flavor and viscosity in the finished product.

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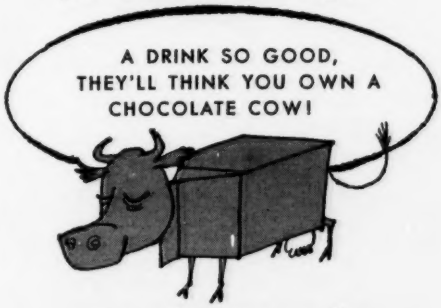

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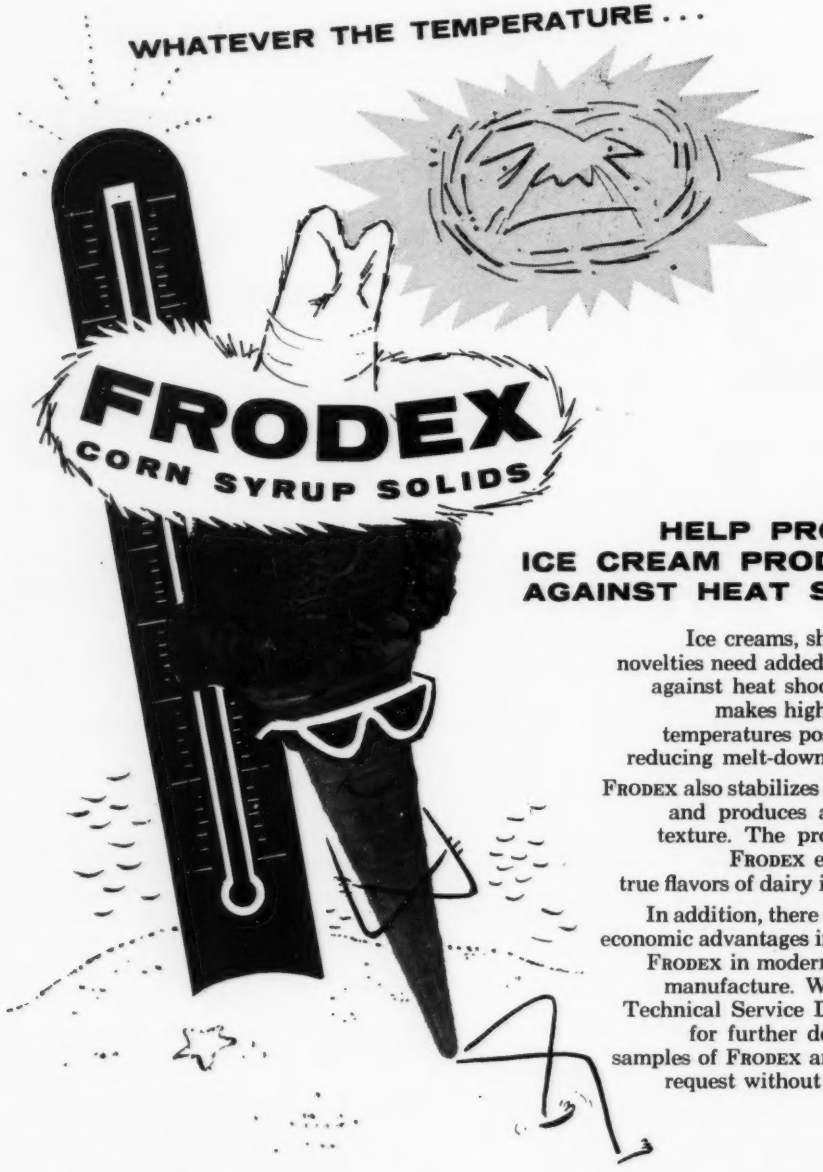

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FRODEX also stabilizes ice crystals and produces a smoother texture. The pro-sugars in FRODEX enhance the true flavors of dairy ingredients.

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"The Factors Affecting the Flavor and Body Characteristics of Mississippi Cheese;" "A Study of Lactose Precipitation in Highly Concentrated Skimmilk;" "The Effect of Various Levels of Solids-not-fat on the Flavor Acceptability of Fluid Milk," and "The Factors Causing Variation in the Protein-Reducing Substances of Milk."

Mr. Herzer established an enviable record with dairy products judging teams coached by him in 17 national contests from 1930-1951. During this time, Mississippi teams placed first in All-Products four times, placed second four times, and won 12 scholarships totaling $10,000.

In 1938, he was elected vice-chairman and in 1939 chairman of the manufacturing section of the A.D.S.A. He was a member of the dairy products judging committee from 1940 to 1956 and a member of the teaching award committee from 1956 to the present. Mr. Herzer served as chairman for the Southern Division of A.D.S.A. in 1948, and he assisted in establishing the Mississippi A.D.A. in 1946. From 1942 to 1955, he was USDA dairy products grader for the state.

Mr. Herzer is also a member of the Association of Southern Agricultural Workers, the Mississippi Farm Bureau Federation, Alpha Zeta, and Phi Kappa Phi.

Recent Deaths

C. ALBERT ALTWEGG, 76, Escondido, Calif., died July 4, 1958, after a short illness. He retired in 1951 after 44 years of association with the Carnation Company.

C. A. Altwegg

It was on Sept. 11, 1906, that Mr. Altwegg went to work in Kent, Wash., for the Pacific Coast Condensed Milk Co., forerunner of Carnation.

Mr. Altwegg was first employed personally by E. A. STUART, founder of the company and, as he wrote when he resigned, "at the princely rate of 16¢ per hour."

When Carnation built its first factory east of the Rockies, located at Richland Center in Wisconsin, he was sent there as superintendent.

In 1915, he was transferred to Oconomowoc, Wis., as superintendent of the "showplace" factory there. Later, he was promoted to district superintendent of the Midwestern and Canadian factories for Carnation.

In 1925, Mr. Altwegg was sent to Europe to build and operate a factory in Germany. Then he was made production superintendent for all factories in Europe of General Milk Company (Carnation subsidiary).

He continued in that responsible position through World War II and the postwar re-

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August 6, 1958

Dr. xxxxx xx xxxxxxxx
Department of Dairy Industry
xxxxxxxxxx State University
xxxxxxxxx, xxxxxxxx

Dear Professor xxxxxxxx:

Thank you for your recent inquiry regarding the use of the CP Swept Surface Heat Exchanger. Incidentally, "Swept Surface" is a Creamery Package trade name.

You are correct, heat is exchanged through the cylinder wall and the heating medium does not contact the product. The unit is offered in models for heating with steam, water or other hot fluids, and for cooling by water, sweet-water, brine, glycol solutions, and by direct expansion refrigerants. All models are of sanitary design and come with a stainless steel exterior.

The CP Swept Surface Heat Exchanger is suitable for heating or cooling anything that can be pumped, but its most efficient use is for the more viscous products and/or for extremes of temperatures not easily obtainable with other type heat exchangers. It is used for a variety of food applications from slush-freezing citrus products to sterilizing dairy and other food products for asceptic canning.

We shall be pleased to discuss the equipment and show you how it fits into many processing lines. It may be just the heat exchanger to open the way for a practical application of the new process which looks possible from your research studies.

We shall look forward to seeing you August 18th.

Very sincerely yours,

Horace Mitten

H. L. Mitten, Jr.
Technical Service Director

HLM:jk

construction period, returning to the United States and the home office of Carnation in Los Angeles in 1949.

Mr. Altwegg was born in Basel, Switzerland, on April 5, 1882. He was educated in the public schools of Basel and then in Swiss agricultural schools, after which he did agricultural work in Germany, in the years 1902–1904. Then he emigrated to the United States. His first job in this country was on a farm at Forrest, Ill., working for $20 a month.

In the fall of that year, he started taking graduate work in dairy science at the Univ. of Wisconsin. And after that he worked for various small dairy concerns in the Midwest until he made his way to the Pacific Northwest and found his life's work with the small and young company that was to become Carnation.

He is survived by his widow, the former M. Antoinette Perrault, who formerly worked in the medical unit of the Carnation general offices in Los Angeles; a son, C. Albert Altwegg, Jr., Arlington, Texas; two brothers, Wilhelm and Fritz Altwegg of Basel, Switzerland, and two grandchildren.

W. R. Davey, 36, assistant professor of dairy science at Pennsylvania State Univ., died June 15 at the National Institutes of Health in Bethesda, Md., following a six-week illness.

W. R. Davey

He was born in Pen Argyl, Pa., and was graduated from Pennsylvania State Univ. in 1942. After serving with the 101st Airborne Division as a First Lieutenant in the Pacific Theatre in World War II, he returned to Penn State to continue his studies. He completed his M.S. and Ph.D. degrees (the latter in 1955) while serving as an instructor in dairy science.

Dr. Davey taught courses in dairy science at Penn State and contributed to the research program in the field of dairy testing. He was adviser to dairy students and served as adviser to the Dairy Science Club of the department for nearly ten years. For many years he served as adviser to the local chapter of Alpha Gamma Rho and was very much interested in student programs. He was a member of Gamma Sigma Delta and Sigma Xi honorary fraternities.

Dr. Davey's activities were uninterrupted up to 6 wk. before his death. The suddenness of his being stricken was a great shock to his associates and his students, with whom he was extremely popular and respected. He is survived by his widow, Mary Catherine, and two daughters.

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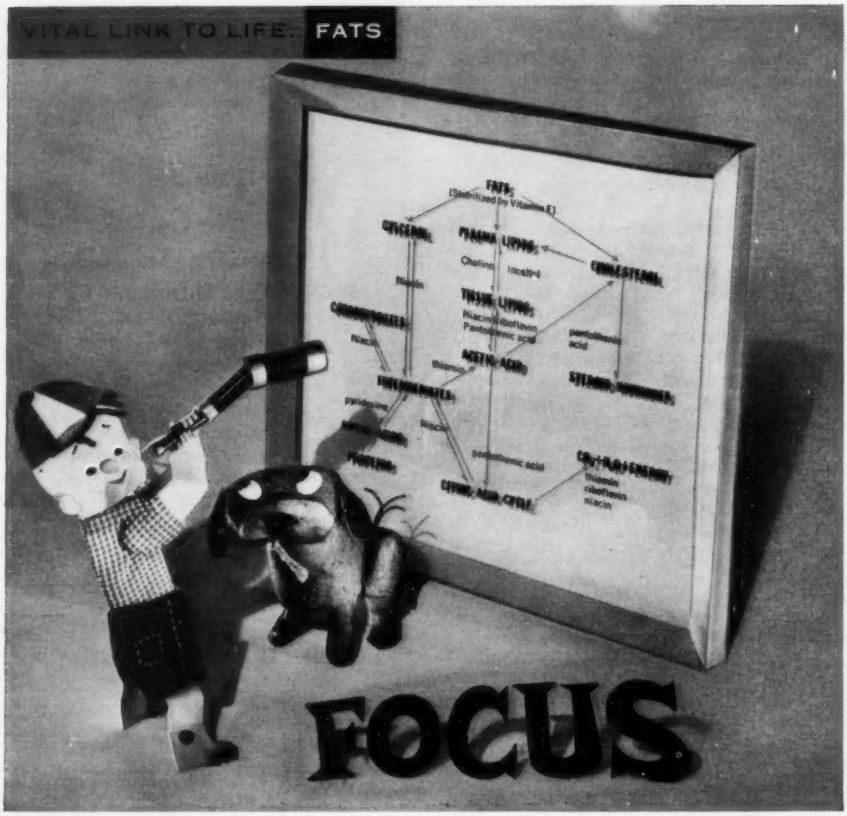

Acetic acid . . . a fatty acid . . . plays a central role in the interaction of nutrients required for synthesis of body tissues . . . maintenance of body warmth . . . and performance of work.

Fats, carbohydrates and proteins all contribute to the metabolic pool of acetic acid which can then be oxidized to carbon dioxide and water with release of energy for body use . . . or can be used for the synthesis of body substances such as cholesterol . . . hormones . . . tissue proteins . . . and fat for storage depots. Vitamins, minerals and proteins in enzyme systems cooperate to sever or join the chemical entities which make up functioning body tissues and yield energy as needed:

Vitamin E protects fats from undesirable oxidation . . . Choline aids the transfer of lipid material from blood to tissues . . . Niacin, riboflavin and pantothenic acid help change tissue lipids to acetic acid and thence to cholesterol and steroid hormones . . . or to tissue proteins . . . or to blood glucose . . . or to energy . . . Thiamine, riboflavin and niacin function in enzymes which release energy from fats, carbohydrates and proteins . . . Pyridoxine is necessary for synthesis of amino acids and body proteins.

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Cornell Cheese Conference to Be Held Oct. 7–9

A Conference on "Fundamentals and Scientific Advances in Cottage and Cheddar Cheese Making" will be held Oct. 7–9, 1958, at Cornell Univ., Ithaca, N. Y.

A high light of the conference will be the presentation of principles and operating details of a newly developed New York State mechanized process for the manufacture of Cheddar cheese, Ched-O-Matic, followed by actual demonstrations on a commercial level.

A comprehensive program dealing with fundamentals and new research in Cottage cheese manufacture has been developed. Included among the many subjects will be the following: Practical measures to insure freshness in cottage cheese; fragmentation studies of large-curd cottage cheese; proper packaging of cheese; modern sanitizing techniques; application of radioactive compounds to cheese-ripening phenomena; quality and yield of cottage cheese made from spray dry milks. A tour of one of the newest and most modern cottage cheese plants in the world is on the agenda.

During one evening the American Cottage Cheese Institute, organized at Cornell in 1956, will hold a dinner and business meeting.

Industry members who will assist the Cornell staff in organizing the conference are C. B. LANE, director of research, Breakstone Foods Company, and ERICK LUNDSTEDT, director of new product development, H. P. Hood & Sons. Among guest speakers will be AL KINNEY, Dixie Cup Corporation; HENRY LEBER, Dairymen's League Co-operative Association, and JIM WILLSON, Klenzade Corporation.

Interested persons from universities and industry are cordially invited. For registration forms, or more information, requists should be sent to F. V. KOSIKOWSKI, Dept. of Dairy Industry, Cornell Univ., Ithaca, New York.

Milk Concentrates Meeting Is Oct. 15–16

The third Milk Concentrates Conference will be held Oct. 15–16 at the Univ. of Wisconsin, Madison.

Main speakers and their topics are C. A. ZITTLE, Eastern Utilization Branch, USDA, Philadelphia, "Factors Affecting the Stability of Milk Proteins"; M. KEENEY, Univ. of Maryland, College Park, "Browning Reaction as Related to Dairy Products"; W. R. MARSHALL, JR., Univ. of Wisconsin,, "Fundamentals of Spray Dryer Design"; N. C. ACETO, Eastern Utilization Branch, USDA, Philadelphia, "Recent Progress in Foam Dried Whole Milk Research: Phases in the Engineering and Development Laboratory"; M. J. PALLANSCH, Eastern Utilization Branch, USDA, Washington, D.C., "Recent Progress in Foam Dried Whole Milk Research: Phases in the Dairy and Meat Laboratory"; S. T. COULTER, Univ. of

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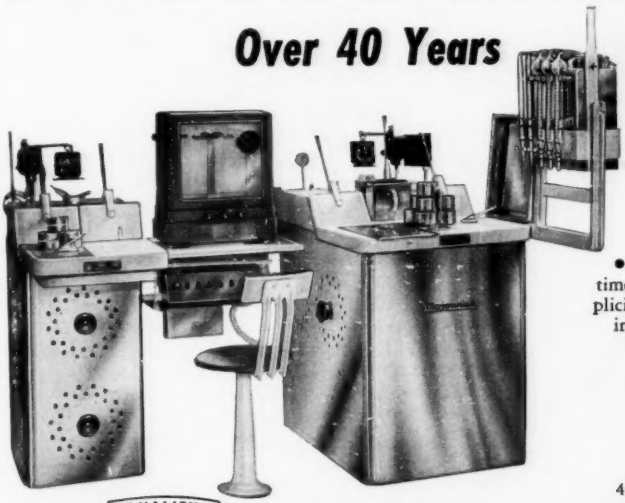

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Minnesota, St. Paul, "Some Current Problems in the Dry Milk Industry"; D. R. PERYAM, Quartmaster Food and Container Institute for the Armed Forces, Chicago, Ill., "Sensory Testing—Science or Applied Art"; M. W. BESSERT, Oscar Mayer and Co., Madison, Wis., "The Use of Sensory Testing Techniques in Research and Product Control"; R. C. LAKAMP, Kroger Food Foundation, Cincinnati, O., "Methods of Evaluating Consumer Preferences"; L. C. THOMSEN, Univ. of Wisconsin, "Production of Quality Process Steam"; W. D. RAMAGE, Western Utilization Research Branch, USDA, Albany, Calif., "Use of Direct Steam in Food Processing"; and A. E. PECH, Food Machinery Co., San Jose, Calif., "Thermodynamics of Milk Heating and Sterilization."

Soviet Agriculturalists Tour U. S.

A delegation of seven U.S.S.R. specialists in animal husbandry has completed a visit to the Animal Husbandry Research Division of the USDA, Beltsville, Md., and will tour eight other U. S. agricultural institutions: North Carolina State College, Raleigh; Univ. of Georgia, Athens; Fort Reno Livestock Experiment Station, Fort Reno, Okla.; Iowa State College, Ames; Iowa State Fair, Des Moines; the Animal Disease Eradication Division of the USDA, Omaha, Neb.; the Univ. of Wisconsin, Madison, and the American Breeders Service, Chicago, Ill.

Georgia News

O. T. FOSGATE spent the summer doing research in physiology and nutrition at the Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn.

J. E. MCGOWAN has resigned his position with the Agricultural Extension Service in charge of the Dairy Herd Improvement Program to become manager of the State Hospital Farm, Milledgeville, Ga. His successor is W. H. MCKINNEY, formerly County Agent at Covington, Ga.

The following dairy conferences and short courses have been announced: Production Short Course, Jan. 27–29, 1959; Annual Meeting of Georgia Chapter of International Association of Milk and Food Sanitarians and Sanitation Conference, Oct. 28–29, 1958.

H. B. HENDERSON will present a paper on "The Influence of the Bulk Tank Program on the Dairy Industry of the United States" at the International Dairy Congress in London, June 29—July 3, 1959. J. J. SHEURING also will present a paper at the Congress: "Manufacturing, Bacteriological and Sanitary Problems Involved in the Processing and Merchandising of Soft Ice Cream."

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4. Conduct research on new ideas suggested by you.

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News from Borden's

C. W. ERICKSON, formerly merchandising manager and district sales manager of Continental Can Company's plastic container division, has joined the Borden Chemical Company in New York as assistant sales manager of the Resinite Dept.

He will be responsible for the development of new product sales, including vinyl tubing used in the dairy, food, and beverage fields. He also will handle merchandising and sales promotion activties for the department's other industrial products.

A graduate of Duke Univ., Mr. Erickson has had more than 12 yr. of experience in the plastics and synthetics fields.

Ontario Appoints Arnott

D. R. ARNOTT has been appointed assistant professor of dairy science at the Ontario Agricultural College, Guelph, Ontario, Canada. Dr. Arnott was formerly in the Dairy Dept., Purdue Univ., Lafayette, Ind.

Dr. Arnott received his B.S.A. degree from the Univ. of Manitoba, his M.S. degree from the Univ. of Minnesota, and his Ph.D. degree from Pennsylvania State Univ. He will be in charge of the market milk dept. and will undertake various research projects in related fields.

California News

Four two-year-old heifers in the California State Polytechnic College Holstein dairy herd were classified "very good" by Holstein-Friesian Association representatives who classified the college Holstein herd this week. This is the highest rating a two-year-old heifer can receive.

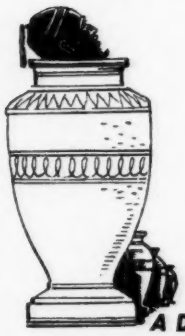
The Holstein herd was classified with an 83.3 point average. Twelve cows were classified "very good," 17 "good plus," and three "good." There were no "excellent," "fair," or "poor" animals.

Two Holstein bulls, Polytechnic Imperial Knight and Polytechnic Imperial Montvic, also were classified "very good." Both bulls were bred at the college.

Rutgers Food Scientist to Head Foundation

W. A. MACLINN, chairman of the Dept. of Food Science at the Agricultural Experiment Station, Rutgers Univ., New Brunswick, N. J., has resigned to become director of the Refrigeration Research Foundation, Colorado Springs, Colo. Among his duties will be the stimulation and encouragement of refrigeration research in university and other laboratories.

A native of Amesbury, Mass., Dr. Maclinn graduated from the Univ. of Massachusetts,



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There are 40 thieves lurking in every business operation today — awaiting every opportunity to rob it of profit. There are Wastes, Production Losses, Distribution Costs and a host of others — most of them well entrenched and not easily recognized.

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The Bureau uses a sharp and practised eye for ferreting out of Milk and Ice Cream operations the 40-odd leaks that may be robbing them of hard-won profit.

If you suspect **you** are paying tribute to some of these hidden thieves, why not join the Bureau **now**? Avail yourself of the Bureau's many services which will put your operation back on the road to **full** profit. For complete information, write

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earning the Ph.D. degree in 1938. He was an assistant professor at Massachusetts for 7 yr.

During the war, he was executive officer and assistant director of the Food and Container Research Institute for the Armed Forces in Chicago and was discharged with the rank of major.

Dr. Maclinn joined the Experiment Station staff in 1946 as associate professor and organized the new Dept. of Food Technology, which was recently renamed the "Dept. of Food Science."

Florida News

E. L. FOUTS and WALTER KRIENKE presented papers at the first Inter-American Food Congress held at Miami Beach, Fla., June 8–12. Dr. Fouts was a member of the General Program Committee and also served as section chairman.

The 1958 Annual Meeting and Convention of the Florida Dairy Association was held at St. Petersburg Beach, June 24–27. Appearing on the program from the Univ. of Florida dairy staff were E. L. FOUTS, E. W. REAVES, WALTER KRIENKE, and HOWARD YOUNG.

Kentucky Short Course to Be Nov. 18–20

The annual Dairy Manufacturing Short Course of the Univ. of Kentucky, Lexington, will be held Nov. 18–20. The short course is sponsored by the Dairy Section of the Univ. of Kentucky and the Kentucky Dairy Products Association.

The course will include the study of ice cream, market milk and cultured products, and cheese. The annual banquet will be held on Wednesday, Nov. 19.

Featured again this year will be clinics for the various dairy products and a milk scoring contest.

Further details concerning the course may be obtained by writing A. W. RUDNICK, Dairy Section, Univ. of Kentucky, Lexington.

Benson, Sen. Wiley Urge "Milk Break"

Sen. ALEXANDER WILEY (R., Wis.) and Secretary of Agriculture EZRA TAFT BENSON have urged the installation of milk-dispensing machines for employees in the Federal Government and in private enterprise.

"Workers in Government and industry should have the opportunity to enjoy drinking milk on their 'breaks,'" the Secretary and Senator Wiley agreed. "A milk break would serve not only as a supplement to the daily diet, but would also smooth jangled nerves in these high-pressure times."

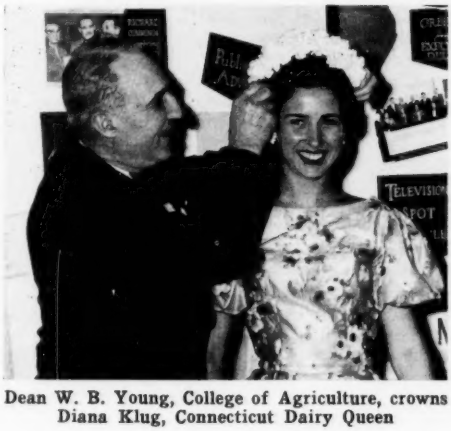
Senator Wiley is a strong supporter of efforts to increase the nation's consumption of dairy products. Secretary Benson, too—in speeches around the country during June Dairy Month—urged the public to drink more milk, eat more cheese, and generally consume more of the healthful dairy foods.

STUDENT CHAPTER NEWS

J. E. JOHNSTON, *Editor*

Connecticut Dairy Club News

The eleventh annual Dairy Club Breakfast was held May 3 at Storrs. At this event the Dairy Club again honored two outstanding contributors to the dairy industry in the State of Connecticut. This year, Adrian Wadsworth, a dairy farmer and president of the Connecticut Milk Producer's Association, and Chris Knudsen, a dairy plant operator, were the honored guests. Their pictures will be permanently hung in the Animal Industries Building. The Dairy Club Yearbook was circulated at the Breakfast and was dedicated to this year's honored guests.



Dean W. B. Young, College of Agriculture, crowns Diana Klug, Connecticut Dairy Queen

The Breakfast was attended by approximately 250 members of the dairy industry, alumni, club members, and University staff. Featured at the event was the crowning of Diana Klug as Connecticut Dairy Queen. Miss Klug was chosen from among five finalists at the Breakfast by a majority vote of those present. She appeared later at functions throughout the state in conjunction with "June Dairy Month" and on the radio and television programs sponsored by the Connecticut Milk for Health Program.

In the afternoon following the Breakfast, the Dairy Club sponsored the annual Fitting and Showing Contest. The show is open to any University student desiring to fit and show dairy animals. The Dairy Queen presented prizes and ribbons to the winners in the various classes. Grand champion of the show was T. Brockett, with a Holstein heifer, and reserve champion was L. Tripp, with a Guernsey heifer. The show classes were interspersed with faculty-student milking and can-rolling contests, with both contests being won by the faculty, as usual.

Minnesota Dairy Science Club News

Over 175 high-school students and their advisers attended the fourth annual Dairy Industry Career Day held on the campus, March 29. Donald Benning, dairy industry senior, was one of the career-day speakers. Benning, past-president of the Dairy Science Club, has been awarded an Alpha Zeta traveling scholarship and will represent the club at the annual meeting of the American Dairy Science Association at Raleigh, North Carolina.

Mr. Carl Mattson, of Land O'Lakes Creameries, spoke on "Problems of the Dairy Industry" at the club's April meeting.

Following the business meeting on May 6, club members were guests of District 25 of the Minnesota Creamery Operators' and Managers' Association at a recognition program honoring members of the Dairy Department staff for their service to industry. Sydney Berde, Assistant Attorney General for Minnesota, spoke on "Milk Marketing Barriers."

The Dairy Department Picnic was held May 27 at Como Park. Over 125 persons attended. The softball game, ably umpired by Dr. S. T. Coulter, proved a real upset, the undergrads upsetting the graduate students and faculty by a score of 7 to 2.

The club softball team, under the direction of Gerald Bratland and Sheldon Erickson, finished the season in second place, with a record of three wins and two losses. The team picked up consolation honors by trouncing Independent Men's Coop, 16 to 10.

Student Chapter Annual Reports

Each student chapter is required to submit an annual report outlining the chapter activities for the year. Your editor was requested at the annual meeting to review and summarize them for this year. The reports were more variable in content this year than in the past, probably because no outline of the information desired had been distributed. However, nearly all of them contained ample evidence that the

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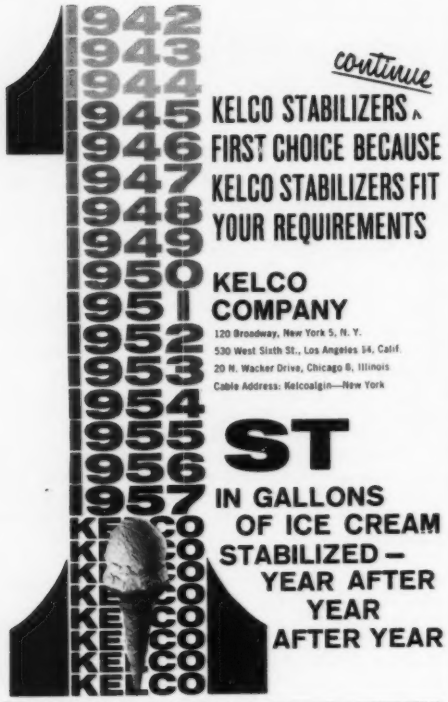

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student chapters have been doing a fine job. Some of the activities reported by the various chapters are listed below, under appropriate headings.

Educational Programs—Guest speakers discussed topics such as agricultural journalism, advertising, foreign dairying, radioactivity in milk, marketing, milk cooperatives, and job opportunities; movies; round-table discussions; tours of farms and plants; sponsorship of judging teams and student fitting, showing, and judging contests; preparation of booths, floats, and other exhibits describing some phase of the industry.

Encouragement of Interest in Dairying as a Career—Participation in Dairy Day and campus Open House activities; sponsorship of 4-H and F.F.A. judging contests; fall welcoming programs for freshmen and other new students on campus; one chapter publishes a booklet for distribution to prospective students, listing the advantages of dairying as a career.

Relations with Alumni and Industry—Assistance with campus conferences and short courses for industrial and farm groups; assistance with shows and sales sponsored by breed associations and other organizations; honoring of outstanding leaders of the dairy industry of the state; publication of newsletters and annuals; participation in homecoming activities.

Fund-Raising Activities—Annual Christmas cheese sale; preparation of lunches for groups meeting on campus; sale of dairy products and other refreshments at shows, sales, fairs, and other agricultural events; operation of milk and ice cream vending machines on campus; sponsorship of dairy heifer sale or raising and selling a dairy heifer.

Campus and Social Activities—Participation in intramural sports; sponsorship of dances, parties, banquets, and picnics; honoring outstanding students.

Association Activities—Encouragement of student affiliate membership; preparation of scrapbook for exhibit at annual meeting; sending delegate to annual meeting.

The above is only a partial list of the activities cited in the annual reports. However, it does illustrate the broad range of projects which engage the attention of the various chapters. It is obvious that no one chapter can become involved in this many activities; however, some of the less active groups may find useful ideas in the list. One factor which was not brought out in most reports was the distribution of committee assignments among students of the various classes. This is a very important point, since the development of leadership ability should be a principal chapter objective. This trait can best be developed in a student organization by the gradual promotion of the member to more responsible committee positions as he gains experience.

Nearly all chapters were in sound financial positions, as judged from their year-end treasury balances.

BOVINE MASTITIS: A REVIEW

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SUMMARY

The principal organisms associated with mastitis are *Streptococcus agalactiae, Streptococcus uberis,* and *Micrococcus pyogenes.* These organisms usually cause a chronic mastitis and a loss in milk yield with or without the appearance of clinical symptoms. Mastitis due to other organisms is not common, but may be a serious problem in an occasional herd, especially following udder infusions improperly administered. When cultural tests are made on incubated milk, the leucocyte count must be used in interpreting the results, and when made on fresh milk, consideration must be given to the number and kinds of organisms present. *S. agalactiae* can be eradicated from most herds by good sanitation combined with frequent cultural tests and treatment of all infected quarters.

The sanitary measures now in general use are inadequate to prevent new and reinfection with *M. pyogenes* and *S. uberis.* Thus, treatment of infected quarters is of limited value.

Degree of exposure to infection, natural resistance of individuals, age, stage of lactation, and prolonged milking duration influence the rate of udder infections. Abusive machine milking, especially prolonged milking and use of worn or poorly designed teat cup liners, tends to increase the rate of clinical mastitis in infected quarters.

Mastitis reduces the milk yield and shortens the productive life of affected cows, causing an estimated loss of $225,504,000 annually in the United States *(2)*. While it is generally agreed that the immediate cause of mastitis is infection, a difference of opinion exists regarding the relative importance of management (predisposing factors) and infection. Some believe that clinical mastitis occurs only when a phase of management reduces the resistance of the animal, and that the main emphasis in control should be placed on good management. Others believe that mastitis is caused by specific organisms and that control should be based on the prevention and elimination of infection.

There is no question that predisposing factors are important and should be minimized so far as practical conditions permit. It is also essential that consideration be given to specific ways of preventing infection and of eliminating infection when it occurs. To date, such measures have been developed for the control of mastitis due to *Streptococcus agalactiae.* Further research is needed to develop specific measures to aid in the control of other forms of the disease.

Information on predisposing factors, infection, diagnosis, control, and antibiotics in market milk will be presented.[1]

PREDISPOSING FACTORS

Evaluation of the relative importance of any single factor is difficult, because it is usually impossible to observe the effect of one factor apart from the effect

[1] A complete review is beyond the scope of this communication. The literature cited here has been limited to reports especially concerned with, or representative of findings on, several phases of the mastitis problem. More complete reviews have been published *(87, 149)*. Literature up to 1938 was abstracted by Munch-Petersen *(107)*, and during 1953 to 1955 by Murphy *(114)*.

of others, and because individual animals under apparently identical conditions differ in susceptibility. Furthermore, most of the literature deals with clinical observations which are difficult to evaluate.

Chilling. Exposure of the udder to chilling probably increases inflammation in udders already infected; however, this has not been determined experimentally. Reid *(169)* observed an increased rate of clinical mastitis in the spring and in the fall in herds in which animals were left in pasture over night while the ground was cold, and in animals which were exposed to drafts in poorly ventilated barns. The relation of new *S. agalactiae* infection to the season of the year was determined in 14 herds (average 755 cows) in Scotland by Ineson and Cunningham *(68)*. The number of new infections that occurred during the spring, summer, fall, and winter was 54, 45, 75, and 38, respectively. The peak months of the year were October and April. The stage of lactation appeared to have no effect on the occurrence of new infection. Oliver *et al. (135)* observed no seasonal trends in the incidence of udder infection and mastitis when allowance was made for the effect of stage of lactation and age differences of spring-autumn–calving cows.

Feed. Observations of Udall and Johnson *(223)* and conversations with dairymen suggest that reducing the amount of protein concentrates in the ration tends to reduce clinical evidence of mastitis in cows with a past history of mastitis and in cows with udder infections, as shown by laboratory tests. However, the limited experiments of Hotis and Woodward *(59)* and Moore *et al. (104)* failed to show a relation between the diets used and either the severity or rate of mastitis. Pounden and associates *(164-166)* found that the diet of cows affected the ability of their milk to resist acid formation by *S. agalactiae* in in vitro tests. The relation of this characteristic to susceptibility or resistance of the udder to infection was not determined. Their findings are of interest and would seem to warrant further studies on the influence of the ration on mastitis. However, definitive experiments would be difficult to arrange, due to other factors that affect the establishment of infection and rate of clinical mastitis in infected quarters.

Hypersensitivity. In the transmission experiments of Jones and Little *(73, 81)*, repeated exposure to small numbers of *S. agalactiae* organisms was required to produce infection. Exposure was accomplished by dipping the end of a small glass rod into dilutions of a 6-hr. broth culture, and passing the end through the teat canal. They suggested that the udder first must be sensitized before infection can result from exposure to small numbers of organisms. This explanation seems to be invalid in view of the findings of Murphy and Stuart *(120)*, that all of 11 quarters of four first-calf heifers became infected following the introduction of 35 ± 31 *S. agalactiae* organisms into the teat cavity by means of a special pipette.

Spencer and Angevine *(199)* reviewed the literature on hypersensitivity and showed that cows may be sensitized to *S. agalactiae* antigens. Intradermal injections of formalinized cultures produced larger and more persistent swellings in infected than in normal cows. Injection of antigens into quarters in which

infection was previously removed by treatment caused the quarters to become swollen and the secretion abnormal 5 hr. later. Control quarters showed a less severe reaction. In a later report, Spencer and Simon *(204)* found that prior sensitization by subcutaneous injections of living organisms did not increase susceptibility to experimental exposure by intramammary infusion of 1,100–24,000 organisms.

While hypersensitivity may not be a prerequisite to infection with *S. agalactiae*, it appears to be involved in the pathogenesis of mastitis due to this organism *(199)*.

Heredity. Data collected on individual herds have shown that selected cow families (dam and daughters) appear to have a relatively high or low incidence of mastitis *(88, 118, 169)*. Milk yield is influenced by heredity, but the volume of milk produced does not appear to be related to susceptibility to mastitis *(118, 169, 235)*. Milking rate is inherited *(29)*, and Dodd and Neave *(31)* found that fast milkers tend to become infected more readily than slow milkers. However, they state that differences in milking rates can not account for the large differences in mastitis-incidence rates found between separate herds. Evidence that the sire is a factor in inherited resistance to infection was given by Legates and Grinnells *(79)* and by Reid *(169)*.

Age. As would be expected, the rate of udder infection increases with age. For example, Seelemann *(187)* reported an infection rate among 5,834 cows of 9, 30, 42, 44, and 52% for animals in their first, second, third, fourth, and fifth lactations; Plastridge *et al. (153)* found that the rate of infection (mostly *S. agalactiae*) increased from 14 to 100%, and that the proportion of animals giving visibly abnormal milk intermittently increased from 8 to 86% from the first to eighth lactation when the animals in two herds were grouped according to lactation. With few exceptions, first-calf heifers are free of *S. agalactiae* at the time of parturition *(87)*. The exceptions are due to infection acquired during calfhood, when calves are fed infected milk and allowed to suckle each other *(172a)*.

An increase-with-age pattern for *S. agalactiae* commonly has been observed; however, different explanations have been proposed. The observations made by Murphy *(112)* on a large herd over a 7-yr. period indicate that an "age factor" independent of teat injury, milking rate, prior sensitization, and degree of exposure is involved. Support for this explanation is given by the findings of Lancaster and Stuart *(76)*. They found that two of seven first-calf heifers, four of five second-calf heifers, and six of six older cows became infected within a period of 15 wk. when exposed by milking them with hands previously dipped in *S. agalactiae*–infected milk. While an age factor may be involved, the observations of Ormsbee and Schalm *(136)*, Plastridge *et al. (151)*, and Spencer and Kraft *(200)* show that the degree and extent of exposure are major factors affecting the rate of infection in heifers as well as in older cows.

An increase of infection with non–*S. agalactiae* streptococci and hemolytic staphylococci, and clinical mastitis, were considered to be due to persisting infections and reinfections, in a herd observed by Oliver *et al. (132, 135)* for

age incidence of infection. In a large *S. agalactiae*–free herd, Schalm and Woods *(185)* found that the incidence of *Micrococcus pyogenes* infection increased with age, from 20% for first-lactation animals to 74% for cows in their eighth lactation.

While the degree and extent of exposure, and persistence of infection, contribute to the relationship of age to incidence of infection, the findings of Murphy *(112)* and Lancaster and Stuart *(76)* indicate that unknown changes associated with age may be related to susceptibility to *S. agalactiae* infection.

Stage of lactation. *S. agalactiae* infection does not appear to be related to the stage of lactation, as indicated by the findings of Plastridge *et al. (151)*. Of 157 heifers in six *S. agalactiae*–infected herds, seven, eight, and seven became infected during the first third, second third, and last third of their first lactation.

Oliver *et al. (133, 135)* reported that the rate of new infection with non–*S. agalactiae* streptococci and staphylococci was highest during the first month of lactation and during the early "dry period". About one-half of the latter infections persisted until calving *(134)*. The rate of infection during the dry period appeared to be related to the milk yield at the last milking and to the method of stripping before the cows were dried off *(133)*. The percentage of quarters that became infected increased from 18% for cows that yielded less than 7 lb. at their last milking to 43% for those that yielded 21 lb. The percentage of new infection during the dry period was 18% for machine-stripped cows and 37% for hand-stripped cows.

Degree of exposure. A major factor in determining the incidence of *S. agalactiae*, and probably other infections in a given herd, is the degree and extent of exposure. This is illustrated by results reported by Spencer and Kraft *(200)*. In two herds with a low incidence of *S. agalactiae* infection (average of 21%), the rate of infection for first-, second-, third-, and fourth-lactation animals was 5.3, 15, 25, and 35%, respectively. In comparison, in ten herds with a high rate of infection (average of 71%), the rate of infection for the four lactations was 61, 68, 68 and 73%, respectively.

Teat characteristics. Injuries resulting from cows stepping on teats, cuts from barbed wire, teat surgery, and lesions at the end of the teat, destroy or impair a natural barrier to infection and may facilitate the entrance into the udder of various kinds of microorganisms present on the skin of the teat. The variety of organisms associated with teat injury was shown by the findings of Ferguson *(43)*. Of 283 samples from cows with mastitis following teat injury, 24% contained *S. agalactiae*, 23% *Streptococcus dysgalactiae*, 15% *Streptococcus uberis*, 10% other streptococci, 15% staphylococci, 6% *Corynebacterium pyogenes*, 2% coliforms, and 7% mixed infections. *S. agalactiae* was not found after injury in herds known to be free from this organism.

The size and shape of the teat, length of the teat canal, and superficial sores on the teats do not appear to be related to the incidence of mastitis *(75, 93, 125, 212)*.

The rate of milk flow (teat patency) appears to be a factor in susceptibility to infection, especially with hemolytic staphylococci *(31, 42, 65, 93, 111, 112, 212)*.

In observations on a herd of 180 cows, McEwen and Cooper *(93)* found that the percentage of animals yielding milk with clots associated with "bacterial infection" was 18% for "hard milkers", 15% for "moderate milkers", and 33% for "easy milkers". Dodd and Neave *(31)* divided 94 first-lactation animals, in a herd with a low incidence of *S. agalactiae* and a high incidence of staphylococcic infection, into five groups on the basis of rate of milk flow. The mean peak flow (lb. per mintue) was 2.4, 3.6, 4.5, 5.6, and 6.8. The rate of new infection with *S. agalactiae* in the five groups was 0.0, 6.6, 3.8, 25, and 30%, and for hemolytic staphylococci, 5, 33, 46, 63, and 67%, respectively. They suggested that either a large or slack teat sphincter, or leaving the machine on longer than necessary for the fast milkers, may facilitate passage of bacteria into the udder.

The soft keratin in the external portion of the teat canal appears to be a natural defense mechanism against infection. Fincher *et al. (45)* found that infection invariably followed swab exposure to *S. agalactiae*, when this material was removed from the teats of heifers that had previously resisted exposure.

Incomplete milking. Leaving 1 lb. or less of milk in the udder does not appear to be a factor in mastitis *(30, 179)*. Schalm and Mead *(179)* found that leaving about 2 lb. of milk did not cause alterations in the milk of normal quarters; however, in *S. agalactiae*–infected quarters an increase in the leucocyte count and visible symptoms of mastitis occurred.

Hand vs. machine milking. Early reports *(19)* indicated a higher incidence of mastitis in machine-milked animals than in animals milked by hand. Recent findings, that the milkers' hands can be a major factor in the spread of udder infections, tend to reverse this situation, providing machine milking is done properly.

Wilson *(231)* reviewed the work of Lancaster and Stuart, in which 12 of 18 cows became infected within a period of 15 wk. when milked with hands previously dipped in *S. agalactiae*–infected milk. The 12 animals were then freed of infection by treatment with penicillin. When milked by machine, with the teat cup clusters dipped in infected milk before being put on each cow, only six of the 18 animals became infected during a similar period. Wilson *(231)* reported that the percentage of herds with *S. agalactiae infection* before and after treatment of infected cows was as follows: for 273 hand-milked herds, 63% pre-treatment and 14% post-treatment; for 122 machine-milked and hand-stripped herds, 68 and 29.7%; and for 189 machine-milked and machine-stripped herds, 52.8 and 8%.

The rate of new infection (mostly non–*S. agalactiae* streptococci) during the early dry period was found by Oliver *et al. (133)* to be 37.2% for a group of hand-stripped cows and 18.3% for a group of cows that were machine-stripped.

Type of milking machine. The type of machine does not appear to be important in mastitis control. Data collected by Ward *(225)* on 1,099 New Zealand herds in which five types of machines were used, failed to show any significant relation between the type of machine and incidence of mastitis. Hodges *et al. (57)* state that "The writers have seen many dairymen in trouble with mastitis

regardless of the type of machine used. Many of these same dairymen using the same machine have reduced mastitis to the point where it is not a serious economic problem. This has, in general, been accomplished through adequate and judicious diagnosis and treatment, coupled with strict attention to good management and sanitation that includes proper milking practices and proper vacuum and pulsation adjustments on the machine."

Type of teat cup liners. In England, "stretched" or "extended" liners and "moulded" liners have been compared in several *S. agalactiae*–free herds. Neave *et al. (129)* observed a reduction in the rate of clinical mastitis in three of four herds when the liners were changed from "moulded type 1" to "moulded type 2" and an increase in one herd after changing from "stretched liner type A" to "moulded type 1". Most of the clinical cases were associated with hemolytic staphylococci.

In 84 cows with a mean age of 3.8 lactations, Dodd *et al. (32)* found that the percentage of clinical mastitis in the quarters of all cows was 5.5% for an extended type liner and 12.7% for a moulded type; and in quarters infected with hemolytic staphylococci and non–*S. agalactiae* streptococci, 9.8 and 37%, respectively. The superiority of the extended liner was attributed to its greater elasticity. No significant difference between the two liners in respect to rate of infection, teat condition, and leucocyte count of normal quarters was observed. They suggest that while the action of a liner may not affect a normal quarter, it can irritate an infected quarter and thus precipitate an attack of mastitis.

Vacuum level. In a review of machine milking and mastitis by Burkey and Sanders *(19)*, the data presented suggested that a vacuum level of over 15 in. of mercury tends to increase the incidence of mastitis. An example of mastitis due to an excessive vacuum was given by Neave *et al. (130)*. In a *S. agalactiae*–free herd of 60 milking Shorthorns a severe outbreak of clinical mastitis involving 26 cows occurred in the spring of 1943. At the time of the outbreak, the vacuum gauge registered 15 in. when, in fact, the vacuum was 19½ in. After replacing the gauge and adjusting the vacuum to 15 in., no further cases were reported. The symptoms were accompanied by distinct lameness and swelling, induration and pain in the affected quarters, and in 12 cows the secretion was coffee-colored with viscid clots. The majority of the bacteria isolated were *S. uberis* and a few were *Streptococcus fecalis,* both of which commonly occur on the skin of teats. A few samples contained no significant organisms.

Evidence that vacuum levels per se within a range of 10, 12, and 17 in. of mercury do not cause mastitis was given in a series of papers by Mochrie and associates *(101, 102, 103)*. No significant differences were seen in the leucocyte count, chloride content, pH values, and incidence of either clinical mastitis or infection, of first lactation heifers and cows milked at the three vacuum levels. All of the animals used were free from *S. agalactiae.*

Milking duration. Evidence that leaving the machine attached to the udder after milk flow has practically ceased contributes to mastitis was presented in the review by Burkey and Sanders *(19)* and in several subsequent reports.

In the observations of Mochrie *et al. (103)* the average leucocyte counts of the milk from cows milked for a normal, twice normal, and four times normal milking duration were 461, 761, and 1,130 thousand per milliliter, respectively; the average chloride values were 131.7, 147.1, and 163.9 mg. %. Although no clinical mastitis developed during the 6 wk. of observation, the increased chloride and leucocyte values are evidence that irritation occurred when the milking time was extended.

The effect of a 4-min. duration of milking and an 8-min. duration on heifers in four herds was observed by Dodd *et al. (30)*. The heifers were milked for an entire lactation. Of 19 animals in the 4-min. group, four became infected and none showed clinical evidence of mastitis. In the 8-min. group of 19 animals, seven became infected and four showed clinical evidence of mastitis in one or more quarters on one or more occasions. Animals were classed as infected when quarter samples contained "pathogenic bacteria" on two or more consecutive tests and showed a reaction to the Whiteside test. Presumably, the infecting organisms were mostly hemolytic staphylococci, since three of the herds were free of *S. agalactiae*.

Evidence that leaving the machine on after milk flow has stopped facilitates passage of bacteria through the teat canal was presented by McEwen and Samuel *(94)*.

Pier *et al. (146)* showed by radiographic means that tissues of the mammary gland can be injured when the machine is allowed to remain on the udder after milking has been completed.

Improvement in milking-machine design to provide visual milking, and vacuum reduction with each pulsation, is being tried by Schalm and Noorlander *(181)*.

Comment. The reports reviewed indicate that: *(a)* The degree and extent of exposure, and milking duration, can affect the rate of udder infections, *(b)* abusive milking practices, exposure of the udder to chilling, and excessive feeding of protein concentrates may increase the incidence of clinical mastitis in infected quarters, and *(c)* the rate of milk flow, stage of lactation (infection other than *S. agalactiae*), age, and heredity influence susceptibility to infection.

INFECTION

During 1874 to 1878, Roberts *(170)* and Lister *(80)* advanced the theory that milk within the healthy udder is germ-free. This was soon followed by the theory that the udder is inhabited by a "normal flora" consisting mainly of streptococci, micrococci, and diphtheroids which are always present in the environment of cattle. Early research also showed that streptococci and micrococci are associated with mastitis. Later, tests were developed for identifying the common mastitis streptococci (*S. agalactiae*, *S. dysgalactiae*, and *S. uberis)* and for separating micrococci into pathogenic (*M. pyogenes)* and nonpatho-

genic micrococci.[2] Transmission experiments have shown that the streptococci listed, Lancefield's Group C streptococci, *M. pyogenes*, coliform organisms isolated from cases of mastitis, and *C. pyogenes* are capable of causing mastitis when injected into the teat cistern *(87)*.

Invasion of the udder by microorganisms is by way of the teat canal, with the exception of transfer of organisms by way of the blood stream in diseases in which septicemia occurs. Following invasion, the establishment of infection depends upon the ability of the organism to survive within the udder; and the development of mastitis upon the ability of the organism to produce substances that are toxic to the glandular tissue. The extent of growth of pathogenic organisms and subsequent evidence of mastitis may be influenced by predisposing factors previously mentioned, especially inefficient milking techniques.

Microorganisms may pass through the teat canal either as a result of suction during milking *(82, 94)*, or by growing their way through, as suggested by Murphy and Stuart *(124)*, and as indicated by the observations of Oliver *et al. (133)* that new infections may occur during the dry period. The ability of the teat canal to resist the passage of *S. agalactiae* seems to explain the differences observed in the susceptibility of individual animals to infection when exposed by the Hadley-Wisconsin swab technique *(46, 121, 123, 124)*.

Murphy *(112)* proposed a three-phase concept of udder infection: *Invasion*—passage of organisms into the interior of the udder (may or may not become established); *infection*—organisms become established; and *inflammation*—reaction of the tissues to injury by the organisms or their products. As pointed out by Spencer and McNutt *(203)*, in *S. agalactiae* infection differentiation between infection and inflammation is difficult, since inflammation is present if the invading organism survives. They found that survival of *S. agalactiae* within the udder is invariably accompanied by inflammation which, in the early stages, is usually subclinical. However, in attempting to visualize the genesis of udder infections in general, Murphy's explanation is useful.

Milk yield. In an experiment on transmission of *S. agalactiae* by Davidson *et al. (28)*, six (13 quarters) of 18 cows became infected within a period of 16 wk. The average decrease in yield of the six cows was 10%. Hale *et al. (49)* reported a drop of 10.2% in the average annual yield per cow in 24 herds following the introduction of *S. agalactiae* infection, and an increase of 14.8% in yield per cow in 91 herds following the elimination of this organism.

Crossman *et al. (25)* observed the effect of *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, and *M. pyogenes* on the milk yield of the individual quarters of the cow's udder. Subclinical mastitis (organism present and Whiteside test positive) associated with either streptococci or *M. pyogenes* was found to result in a

[2] In Bergey's *Manual of Determinative Bacteriology*, 7th ed., hemolytic coagulase positive members of the family Micrococcaceae are classed as *Staphylococcus aureus*, and the coagulase negative members either as *Staphylococcus epidermidis* or as species of the genus Micrococcus. However, because the abbreviations for Streptococcus and Staphylococcus are similar, the term *M. pyogenes* will be applied herein to pathogenic staphylococci (hemolytic and/or coagulase positive), and cultures regarded as nonpathogenic will be designated as nonhemolytic or coagulase negative micrococci.

gradual reduction in the proportionate yield of most quarters and in no measurable reduction in a few quarters. A decrease in yield was found to continue after clinical but not bacteriologic cures in infected quarters.

Rate of infection. Numerous organisms have been reported as associated with mastitis. While an uncommon organism may be primarily responsible for mastitis in a given herd, *S. agalactiae*, other streptococci, and *M. pyogenes* are by far the most important causative agents in the cattle population as a whole. This has been shown by surveys made in England *(1, 63, 65, 205, 231)* and in the United States.

In Connecticut, of the 1,191 herds tested during 1957, 650 were *S. agalactiae*–free, either on initial test or as the result of treatment. In the *S. agalactiae*–negative herds (15,407 cows), 14% of the cows were classed as infected with non–*S. agalactiae* streptococci, 18% with *M. pyogenes*, and less than 1% with other organisms. In the 541 *S. agalactiae*–infected herds, 23% were classed as infected with this organism, 8% with other streptococci, 18% with *M. pyogenes*, and less than 1% with other organisms. The higher incidence of non–*S. agalactiae* infection in the *S. agalactiae*–free herds is believed to be apparent rather than real, because an animal with *S. agalactiae* in one quarter and another organism in some other quarter was classed as *S. agalactiae*–infected.

In New York State *(44, 106)*, in "first A surveys" on 53,493 cows, the percentage of cows with abnormal secretion was 16.6%, the percentage of quarters infected with *S. agalactiae* was 10%, other streptococci 6%, *M. pyogenes* 8%, and other organisms, less than 1% (excluding nonhemolytic micrococci and diphtheroids other than *C. pyogenes*). In the "last A survey" on 39,043 cows, the percentage of cows with abnormal secretion was 9%, and the percentage of quarters infected with *S. agalactiae* was 5%, other streptococci 7%, *M. pyogenes* 8%, and other organisms, less than 1%.

Assuming that an average of two quarters per cow are infected, the results obtained in both states show that in *S. agalactiae*–infected herds about 50% of the cows and 25% of the quarters are infected with one of the three principal causative agents in mastitis, and that reduction of *S. agalactiae* infection through treatment does not necessarily result in a significant increase in other types of infection. The New York surveys also show that a reduction in *S. agalactiae* infection was accompanied by a reduction in clinical mastitis.

S. agalactiae belongs to Lancefield's Group B *(77)*. Mastitis due to this organism is contagious. Its habitat is the bovine udder. Apart from surfaces contaminated by infected milk, such as the skin of the teats, milkers' hands, and teat cups it is rarely found outside of the bovine udder *(1)*. The organism occurs occasionally in the human throat; however, for practical purposes this source of possible infection for the cow appears to be of little importance. *S. agalactiae* is highly susceptible to the antibacterial action of penicillin and chlortetracycline. The development of penicillin-resistant strains following treatment has not been observed *(6, 45, 138, 213)*.

The development of infection is usually slow, with clots appearing intermittently in the fore milk and increasing in amount, followed by a gradual in-

duration of the quarter, which may progress until milk secretion ceases *(205)*. As few as 35 ± 31 *S. agalactiae* organisms may produce infection when introduced into the teat cavity *(120)*. Following the entrance of an infective dose into the teat cavity, clinical mastitis may develop within a few days or may be delayed for weeks *(76, 120, 121, 203)*. Once the organism becomes established, the infection tends to go through a cycle of an increased *S. agalactiae* count, followed by an increased leucocyte count, followed by a decreased *S. agalactiae* count, and repetition of the cycle at irregular intervals *(81, 120, 121, 125)*. Clinical symptoms may or may not appear at, or immediately following, the peak of growth activity in the cycle.

Histopathologic findings on *S. agalactiae*–infected glands, producing either clinically normal or abnormal milk, show that acute inflammatory foci occur in from a few alveoli to one or more lobules. These areas later become fibrotic and new areas of inflammation develop, with a corresponding gradual decrease in milk yield *(54, 81, 141, 203, 231)*.

Streptococci other than S. agalactiae. The majority are either *S. uberis* or *S. dysgalactiae*. While these organisms have been isolated from bovine tonsils and vaginae *(87)*, the main reservoir of infection appears to be the infected udder. The literature on biochemical characteristics which differentiate between *S. agalactiae*, *S. uberis*, and *S. dysgalactiae*, and between these organisms and saprophytic streptococci found in the environment of cattle, was reviewed by Little and Plastridge *(87)*. *S. uberis* belongs to none of Lancefield's serologic groups *(77, 162)*, and infected quarters usually show subclinical mastitis unless associated with teat injury *(43)* or with abusive machine milking *(32, 129, 130, 231)*. *S. dysgalactiae* belongs to Lancefield's serologic Group C *(77)*, although the colonies on blood agar are gamma or alpha hemolytic. Mastitis due to this organism may be mild or severe, is usually of short duration, and is not common in most herds. Wide-zone beta hemolytic streptococci belonging to Lancefield's Groups A, C, E, G, and K are rarely associated with mastitis. Group C *(Streptococcus zooepidemicus)* has been found more frequently than Groups A, E, G, and K *(87)*.

Micrococci. The relation of hemolytic and plasma coagulating ability of udder micrococci to evidence of mastitis was first reported by Plastridge *et al. (155, 160, 161)*. Some of the results *(155)* were as follows: The average leucocyte count of 2,125 bacteriologically negative samples was 73,000 per ml.; of 298 samples which contained nonhemolytic micrococci, 220,000, and of 398 samples which contained *M. pyogenes*, 1,361,000. The percentage of samples in the three groups with cell counts in excess of 500,000 per milliliter was 2, 12, and 63, and with cell counts in excess of 1,000,000 per milliliter, the percentage was 0.1, 3, and 43. None of the samples in the first two groups, and 5.2% of those in the latter group, contained visible clots. A close correlation has been found between hemolysis on ox or sheeps' blood agar and ability to coagulate either human or rabbit's blood plasma, and between ability to coagulate plasma and pathogenicity *(93, 155, 178, 185, 197, 198)*. In general, the coagulase test is preferable to hemolysis, or lack of hemolysis, on blood agar for separating micro-

cocci into pathogenic *(M. pyogenes)* and nonpathogenic groups *(178)*.

Nonhemolytic coagulase negative micrococci occur frequently in freshly drawn milk samples, but are seldom associated with clinical mastitis. However, their presence in the udder may cause a mild degree of irritation, which is manifested by a moderate increase in leucocyte count *(93, 155)*. Histologic lesions of mild or moderate intensity have been seen in quarters infected with nonhemolytic micrococci *(54)*.

M. pyogenes. Most of the cultures of bovine origin produce beta toxin only, or beta and alpha toxin *(16, 97, 98, 197)*. The rate of infection was found to be second to that of *S. agalactiae*, in surveys made in Connecticut and New York State. In *S. agalactiae*–free herds they are usually the most common organism associated with both clinical and subclinical mastitis. In general, the degree of inflammation tends to be milder, and the occurrence of clots in the milk from infected quarters less frequent, than in *S. agalactiae* infection *(76, 93, 110, 155, 182, 185)*. However, misuse of the milking machine may increase the rate of clinical mastitis in infected udders *(32, 129, 130, 186)*. First-half heifers are highly susceptible to infection *(182, 206)*, and infection during the dry period may account for mastitis at calving time *(133, 134, 135)*. Evidence that *M. pyogenes* mastitis should be regarded as contagious has been reported *(129, 136)*.

Severe acute mastitis accompanied by a systemic reaction due to infection with highly toxigenic strains of *M. pyogenes (85, 173)* is not common. Such cases are usually sporadic, may result in gangrenous mastitis, and do not respond to intramammary infusions.

The principal source of *M. pyogenes* is essentially other infected glands *(185)*, and the principal extramammary reservoirs are the skin of the teats and teat cups of the milking machine *(202)*. Most cultures are sensitive to penicillin, chlortetracycline, and oxytetracycline *(3, 36)*; however, individual strains may show considerable resistance to one or more of the antibiotics listed *(36, 186)*.

Corynebacteria. Diphtheroids, or members of the genus *Corynebacterium* other than *C. pyogenes*, are often found in freshly drawn milk. They are seldom associated with clinical mastitis. *C. pyogenes* causes a severe acute mastitis which may become gangrenous *(10, 173)*. This form of mastitis is not common in the United States. The disease may occur in pregnant heifers and dry cows as well as in lactating animals *(99)*. *C. pyogenes* is differentiated from other corynebacteria by producing weakly hemolytic colonies on ox blood agar, producing little, if any, growth on nutrient agar, and by producing a soft curd and partial peptonization of litmus milk in five days.

Miscellaneous organisms. A wide variety of microorganisms have been found to be associated with mastitis outbreaks in certain herds. Coliform organisms,[3] *Pseudomonas aeruginosa*, and yeasts are the principal organisms involved. These organisms are resistant to penicillin and there is evidence to indicate that infec-

[3] Bacteria which occur as gram negative, nonmotile rods, and which ferment lactose with acid and gas production, and produce moist, white, raised colonies on agar. The majority of cultures isolated from cases of mastitis belong to the genus Aerobacter, as described in Bergey's *Manual of Determinative Bacteriology*, 7th ed., 1957.

tion in some animals has resulted from lack of aseptic techniques in administering udder infusions.

Coliform organisms may cause either a mild mastitis or a severe acute mastitis with systemic symptoms *(7, 17, 20, 35, 117, 149, 151, 183, 229)*. Coliform organisms responsible for acute mastitis differ from ordinary strains by possessing a distinct capsule *(35, 149)*. The onset of acute coliform mastitis is sudden. Affected cows usually appear normal at one milking and show the following symptoms by the next milking: nearly complete cessation of udder secretion, loss of appetite, depression, a temperature of 104–108° F., and one or more swollen quarters. Usually, one or two cows in a herd are affected; however, in a few herds the disease has spread, within a few months, to 25% of the milking animals *(87)*. It is of interest that death of calves due to capsulated coliform organisms has been reported as occurring simultaneously with an outbreak of mastitis due to these organisms *(229)*.

Mastitis due to *P. aeruginosa* is usually sporadic, but in a few herds may reach serious proportions *(18, 21, 214)*. The onset is usually sudden and accompanied by clinical symptoms of relatively short duration. Infection may persist in a given quarter for several months, with occasional attacks of clinical mastitis. Pseudomonads are common in the environment of cattle. They are highly resistant to disinfectants *(209)* and to penicillin *(214)*, streptomycin *(175)*, chlortetracycline *(176)*, and oxytetracycline *(8)*.

Yeast-like fungi associated with mastitis were first reported by Murphy and Drake *(115)*. Their culture was regarded as a member of the genus Trichosporon. Ten infected quarters were seen during a 6-yr. period in a herd of 120 cows. The milk from nine quarters was macroscopically abnormal for a period of from six to 12 days, and the leucocyte count remained near 1,000,000 per milliliter for the duration of the 69-day period of observation. The cultures grew poorly on blood agar. The disease was reproduced by inoculation of the culture into a quarter of a normal udder. These organisms are resistant to penicillin and streptomycin, and in some herds infection has followed infusions of udders with penicillin preparations *(5, 67, 210, 217)*.

Cryptococcus neoformans mastitis has been reported in two herds *(163, 191)*. The majority of clinically affected animals suffered severe attacks with marked swelling of the udder and decreased milk production. None of the antibiotics tried were effective, and reasonable success in reducing infection was achieved by segregating infected animals, improved sanitation, and care in milking-machine operation. The only detected source of infection observed by Pounden *et al. (163)* was infected udders. Simon *et al. (191)* reported that the administration of an antibiotic preparation without regard to aseptic techniques appeared to be the source of infection. The organism grew on blood agar and Sabouraud's maltose agar and was found to be pathogenic for mice, usually causing focal liver necrosis within 15 days following intraperitoneal inoculation.

Pasteurella multocida mastitis has occurred in a few herds *(9, 140, 218)*. Treatment with chlortetracycline, oxytetracycline, and streptomycin was generally unsatisfactory *(9)*.

Udder infections with acid-fast organisms, other than *Mycobacterium tuberculosis*, following intramammary infusions with antibiotics in oily suspensions, have been observed *(211, 216)*.

Comment. Herd surveys, pathologic changes seen in infected glands, and results of transmission experiments leave no doubt that infection is the primary cause of mastitis. Predisposing factors are considered secondary because, as indicated in the preceding section, they contribute to mastitis mainly by either increasing the chances for infection to occur or by causing clinical attacks of mastitis in infected quarters. In the Connecticut and New York State surveys, about 50% of the cows and 25% of the quarters were found to be infected, and the rate of infection among the cows on initial test was found to be about 22% for *S. agalactiae*, 11% for non–*S. agalactiae* streptococci, 16% for *M. pyogenes*, and less than 1% for other organisms. Infection with streptococci and with *M. pyogenes* may cause either subclinical or clinical mastitis. A decrease in milk yield of quarters infected with these organisms has been shown to occur in the absence of clinical evidence of mastitis.

DIAGNOSIS

Depending on the degree of inflammation at the time of examination, mastitis may be classed as subclinical, mild clinical, and severe clinical. Subclinical mastitis is characterized by changes in the milk, such as, an increased pH value, chloride content, or leucocyte count, in the absence of obvious swelling of the udder or clots in the milk. Mild clinical mastitis is characterized by a slight or moderate swelling or induration of one or more quarters and/or a visibly abnormal secretion, including clots revealed by use of the strip-cup. In severe mastitis, the udder is swollen, the secretion is grossly abnormal, and the animal may show an increase in body temperature, loss of appetite, depression, and nearly complete cessation of milk secretion. The latter form of mastitis is not common and is usually caused by capsulated coliform organisms, *C. pyogenes*, or highly toxigenic strains of *M. pyogenes*.

The term "mastitis" is similar to the term "abortion." Both terms describe an abnormal condition, but fail to indicate the cause. The organisms most commonly associated with mastitis are *S. agalactiae*, non–*S. agalactiae*-streptococci, and *M. pyogenes*. Infection with any of these organisms is usually chronic, with clinical flare-ups occurring at irregular intervals. For this reason, diagnosis should be based on bacteriologic tests combined with tests to detect evidence of inflammation. In the diagnosis of *S. agalactiae* mastitis for control purposes, tests which approach 100% efficiency are required. In the other forms of mastitis, laboratory tests are useful in *(a)* selecting the antibiotic most likely to be effective in cases of clinical mastitis, *(b)* culling chronically infected animals, and *(c)* indicating the level of udder health in a herd.

Tests for evidence of inflammation. Barn tests that may be used to estimate the extent of mastitis in a herd are: physical examination of the milked-out udder for abnormalities, strip-cup test for clots, bromthymol blue test for altered pH, Whiteside test *(230)* as modified for field use *(109, 116, 177)*, and the

"California Mastitis Test" developed by Schalm and Noorlander *(180)*. In the Whiteside and C.M.T test, the degree of precipitation or gel formation tends to be related to the leucocyte count *(116, 145, 177)*. Schalm and Lasmanis *(178)* reported the following percentages of positive reactions to the Whiteside test: in *S. agalactiae* or *M. pyogenes* infection 50, coliform infection 41, coagulase negative micrococci and non–*S. agalactiae* streptococci infection 23, and "no significant" bacteria, 14. Of the barn tests, the strip cup is most commonly used; however, on the day of examination only about one-half of the *S. agalactiae*–infected quarters *(231)* and about one-fourth of the quarters infected with *M. pyogenes* are detected *(205)*. Of the laboratory tests for evidence of mastitis, the leucocyte count is generally regarded as superior to other procedures. All tests for abnormality of milk give variable results from time to time when applied to chronically infected quarters, may be positive when applied to milk from uninfected cows in early or late lactation, and none indicates the nature of the causative agent.

Leucocyte count. Milk from normal quarters rarely contains more than 500,000 leucocytes per milliliter *(4, 84, 89, 93, 110, 120, 121, 155)* and the milk from infected quarters usually exceeds this number. Hucker *(62)* regarded a count of 500,000 per milliliter as indicative of an abnormal udder condition. Anderson *(4)* reported that the average leucocyte count of milk from quarters infected with *M. pyogenes*, non–*S. agalactiae* streptococci, and *S. agalactiae* was 1,400,000, 1,900,000, and 2,100,000 per milliliter, respectively. In comparison, the average count of 3,789 samples from uninfected quarters was 180,000 per milliliter. McEwen and Cooper *(93)* collected quarter samples at intervals of 3 wk. over a period of 14 mo. from a herd of 180 cows. They found that the percentage of samples with counts of 1,000,000 or more per milliliter was: for quarters infected with *S. agalactiae*, 82.8, *S. dysgalactiae*, 42.9, *S. uberis*, 55.5, *M. pyogenes*, 52.9, nonhemolytic coagulase negative micrococci, 7, corynebacteria (not *C. pyogenes*), 5.8, and no bacteria, 1.2. In the transmission experiments of Little *(81, 82, 83)*, Murphy *(120, 121)*, and Pattison and Holman *(141)*, leucocyte counts of 1,000,000 or more per milliliter invariably followed the establishment of infection with *S. agalactiae*.

After the elimination of infection with streptococci and *M. pyogenes* by treatment, a period of from 1 to 5 wk. is usually required for the leucocyte count to return to normal *(14, 195)*.

Noninfectious factors which affect the leucocyte count. Age, milking practices, and stage of lactation may affect the leucocyte count.

Counts of up to 300,000 per milliliter for heifers and counts of 500,000 to 1,000,000 per milliliter for older cows were considered normal by Little *(83)*. Of 192 samples collected from normal heifers by Mochrie *et al. (102)*, 94% contained less than 300,000 leucocytes per milliliter. In comparison, the average count of milk from uninfected cows with an average of 2.4 lactations was found to be 446,000 per milliliter *(103)*.

Machine-milked normal cows gave milk with an average count of 368,000 per milliliter as compared with a count of 112,000 per milliliter for milk from

hand-milked cows, in observations reported by Cone *(24)*. Although Mochrie *et al. (102)* observed no difference in the average count of milk from heifers that were machine-milked at 10 and 17 in. of vacuum, they *(103)* found that the average count for milk from older cows milked for normal, twice normal, and four times the normal milking duration, was 461,000, 761,000, and 1,130,000 per milliliter, respectively. It seems very unlikely that the latter abuse would occur under practical conditions. The animals used by Mochrie *et al.* were free of infection with streptococci, *M. pyogenes*, and gram-negative bacteria.

From a few days to 2 wk. following calving (varies for individuals), the milk from uninfected cows usually contains from 500,000 to 2,000,000 leucocytes per milliliter. As explained by Hadwen and Gwatkin *(47)*, "The mucinous state of the milk in newly calved cows may call forth large numbers of polymorphonuclear leucocytes which will be found gorged with the mucin; later, both mucin and leucocytes disappear." Murphy's *(110)* observations on 64 uninfected quarters showed that the leucocyte count exceeded 500,000 per milliliter in only 0.5% of 770 samples collected from 1 wk. after freshening for as long as milking was done. McEwen and Cooper *(93)* considered that increased leucocyte counts obtained within 2 wk. after calving and for a period of 4 wk. before drying off were not significant.

Leucocyte count of herd milk. In tests made by MacLeod *et al. (90)* on milk from 31 herds, counts of 1,000,000 or more per milliliter were usually obtained when the percentage of udder infection was 40% or more. Cows were classed as infected when the milk from one or more quarters contained either *S. agalactiae* and 500,000 or more leucocytes per milliliter, or other organisms and 1,000,000 or more leucocytes per milliliter. Factors other than infection apparently affected the count sufficiently to prevent a close correlation between the cell count of herd milk and the rate of infection, when the rate of infection was under 40%. It appears that occurrence of cell counts in excess of 1,000,000 per milliliter in herd milk on more than one occasion would justify an investigation of the possible cause of the high count, including a herd survey to determine the incidence of udder infection and a check of the milking machine and milking practices.

Cultural tests. These are necessary in determining the identity of organisms associated with both subclinical and clinical mastitis. However, organisms found in milk samples, including *S. agalactiae*, may originate from the skin of the teat *(1, 76, 234)* and teat canal *(73, 121)*, as well as from within the udder. Contamination of the sample can be reduced by careful disinfection of the skin of the teat, especially the teat orifice. Leucocyte and colony counts have been used to differentiate between organisms associated with infection and those from the skin of the teat or teat canal. Milk from quarters infected with known mastitis organisms usually contains leucocytes in excess of 1,000,000 per milliliter, as previously shown, and usually more than 200 per milliliter of the infecting organism, as shown by numerous workers *(4, 81, 93, 110, 120, 121, 129, 132, 141, 197)*. Although neither criterion of infection is infallible, both have been shown to be useful in the practical application of cultural tests.

Incubated milk. In some laboratory procedures, milk samples are incubated before culturing, primarily for the purpose of detecting *S. agalactiae.* From the start, it was found necessary to use chemicals in the incubated milk and in the blood agar medium to prevent or retard the growth of bacteria other than *S. agalactiae.* The chemicals most commonly used, alone or in combination, are sodium azide or thallium acetate to retard growth of gram-negative bacteria, and crystal violet to prevent the growth of micrococci *(26, 37, 95, 137, 150, 159, 198, 234).* None of these agents will inhibit growth of fecal streptococci. To separate *S. agalactiae* from these organisms and *S. uberis,* either esculin *(37)* or carbohydrates which are fermented by non–*S. agalactiae* streptococci but not by *S. agalactiae (156, 159)* are included in the blood agar medium, in addition to crystal violet. Each lot of crystal violet should be tested before use to determine the concentration necessary to prevent growth of micrococci without preventing growth of *S. agalactiae.*

Interpretation of the probable significance of organisms isolated from incubated samples requires use of the leucocyte count. In routine tests made in Connecticut *(150, 156, 158, 159),* incubated samples are screened by microscopic examination of ten fields of stained films (0.01 ml. on 1 cm.^{2}). Samples found to contain less than 1,000,000 leucocytes per milliliter and no streptococci are classed as negative; those containing streptococci are cultured on a selective medium for *S. agalactiae,* and those containing no streptococci and 1,000,000 or more leucocytes per milliliter are cultured on blood agar. Weakly hemolytic streptococcus colonies on the selective medium are classed as *S. agalactiae,* colonies surrounded by green or brown zones as non–*S. agalactiae,* and gamma colonies are identified as *S. agalactiae* or non–*S. agalactiae* by the modified CAMP test *(126).* Micrococcus colonies producing hemolytic zones (over 1 mm. in diameter) on blood agar are classed as *M. pyogenes* and others as nonpathogenic. Quarters yielding samples that contain *S. agalactiae* and have a leucocyte count of 500,000 or more per milliliter are classed as *S. agalactiae*–infected. Quarters yielding samples that contain 1,000,000 or more leucocytes per milliliter are classed according to the type of culture obtained. Quarters yielding samples that contain organisms other than *S. agalactiae* and have a leucocyte count under 1,000,000 per milliliter are classed as negative. Samples from cows in early or late lactation, which contain 1,000,000 or more leucocytes per milliliter and which are culturally negative for streptococci and *M. pyogenes,* are classed as negative.

Disadvantages in the culturing of incubated milk are that *(a)* the leucocyte count must be determined, *(b)* two mediums must be used, a selective medium for streptococci and blood agar for *M. pyogenes,* and *(c)* contaminating fecal streptococci and coliform organisms are more likely to prevent the detection of pathogens than when fresh milk is cultured. Advantages are that *(a) S. agalactiae*–infected quarters (leucocyte count of 500,000 or more per milliliter) which at times may yield milk containing fewer than 100 organisms per milliliter *(73, 120, 121)* may be detected; and *(b)* the amount of cultural work can be reduced by culturing only those samples which contain either streptococci or

1,000,000 or more leucocytes per milliliter, as shown by microscopic examination. While *S. agalactiae* can be cultured from some samples in which streptococci are not seen on microscopic examination, the findings of Sethi *et al. (188)* indicate that these organisms usually do not originate from inside the udder.

Hotis test. The test was described by Hotis and Miller *(58)* and is made by mixing 0.5 ml. of a 0.5% solution of bromeresol purple with 9.5 ml. of milk in a test tube, incubating the mixture, and observing the color after 24–48 hr. The appearance of yellow flakes on the side of the tube *(58)* or a thick yellow deposit in the bottom of the tube *(108)* is indicative of the presence of *S. agalactiae.* However, other organisms may produce a similar reaction *(92, 96, 108)* and some samples which contain *S. agalactiae* are not detected *(13, 174).* In routine use of the test, a wide range of color reactions are obtained which are difficult to interpret.

Fresh milk. Strict fore milk should be used because it usually contains more organisms and leucocytes than milk drawn after several streams have been discarded. Blood agar is the medium of choice, because it supports the growth of practically all organisms associated with mastitis, and the colonial characteristics aid in identifying the organisms present. Bovine blood should be used for the detection of hemolysis by *M. pyogenes (97, 98)*; however, the blood from the donor cow must be free from antitoxin.

Gravity cream, sediment from centrifuged samples, and whole milk have been streaked on the surface of blood agar plates, and 1-ml. amounts of diluted milk (1 : 5 to 1 : 20) have been used in preparing pour-plates. In addition, fresh milk samples have been cultured on esculin blood agar *(129, 132),* esculin crystal violet blood agar *(37),* and on this medium plus 1 : 3,000 of thallium acetate *(234).* While the culturing of sediment or the use of pour-plates may be slightly more effective in detecting *S. agalactiae* than culturing whole milk or gravity cream on the surface of blood agar, these procedures are impractical for use in routine tests.

In the New York State mastitis research and control program *(127),* 0.01 ml. of a fresh fore-milk sample is streaked onto one-fourth of the surface of a bovine blood agar plate. The plates are examined after 24 hr. of incubation and a representative streptococcus colony, when present, is inoculated onto an esculin (0.1%) ferric citrate (0.01%) blood agar plate. The streptococci are classed as *S. agalactiae*–type if they do not split esculin, and as non–*S. agalactiae* if they do split esculin. Micrococci are classed as pathogenic or nonpathogenic on the basis of hemolytic ability. Other organisms are tentatively identified by their colonial characteristics. The presence of three or more colonies of the same type are usually considered significant, although true streptococcal infections usually show hundreds of colonies. When contamination sufficient to show by this method occurs, it usually appears as small numbers of several kinds of bacteria. In this case, three or more colonies are not regarded as significant, and an attempt is made to establish whether *S. agalactiae* is present.

Disadvantages of culturing 0.01 ml. of fresh milk on blood agar are *(a)* a few *S. agalactiae*–infected quarters, in which the number of organisms in the sample

is less than 100 per milliliter, may be missed, *(b)* streptococcus colonies on the blood agar require further identification, and *(c)* in some instances, the identification of more than one streptococcus colony may be advisable. Advantages of culturing fresh milk over incubated milk are that *(a)* less interference results from contaminants, *(b)* fewer false positives for *S. agalactiae* infection are obtained, and *(c)* an estimation of the number of organisms present in the sample is possible.

Esculin bovine blood agar also has been used for culturing fresh milk. Oliver *et al. (132)* streaked 0.05 ml. of fresh milk on the surface of the medium, and regarded as positive, samples that yielded "large numbers" of streptococci or *M. pyogenes* and gave a positive Whiteside test. Neave *et al. (129)* inoculated the surface of esculin blood agar with 0.025 ml. of milk and considered the appearance of ten or more colonies of either streptococci or *M. pyogenes* as evidence of infection. The counts of milk from persistently infected quarters were seldom less than 200 per milliliter. Although few of the samples examined by Oliver *et al. (132)* and none of those examined by Neave *et al. (129)* contained *S. agalactiae*, it appears that esculin blood agar may have an advantage over blood agar for the routine culturing of fresh milk, since the esculin should aid in separating *S. agalactiae* from other streptococci.

The method adopted by the Technical Committee of the Agricultural Research Council of England *(213)* for the examination of fresh milk samples was described by Wilson and Slavin *(234)*. A layer of esculin crystal violet blood agar *(37)* plus 1:3,000 of thallium sulfate or thallium acetate is placed in jars of the cold cream type, 4 cm. deep and 6 cm. wide, and equipped with a screw type aluminium lid containing an absorbent pad. The jars are incubated before use to test for sterility and to dry the medium. Small amounts of fore milk from all four quarters are drawn into the jar, spread over the surface of the medium, and the excess discarded. The jars are inverted, incubated 48 hr., and the number and kinds of colonies recorded. Streptococcus colonies are identified by the CAMP test *(23)*. In cows showing clinical mastitis, the sample is drawn as described, but the lid is replaced before discarding the excess milk, and the jar inverted so that the absorbent pad in the lid is soaked with milk and clots. The excess milk is then discarded. These jars are marked to identify cows with clinical mastitis. At the laboratory, material is removed from the pad with a loop and cultured on blood agar to detect mastitis due to organisms other than streptococci. Disadvantages of this procedure are that *(a)* in large-scale testing the size of the jars, as compared with test tubes, would present a problem in handling, refrigeration during transportation, and incubation, *(b)* cows with mastitis due to organisms other than *S. agalactiae*, and which is subclinical at the time of test would not be detected, and *(c)* the infected quarters would not be identified. The advantages claimed for this method are *(a)* a reduction in the cost of examining samples, as compared with most other methods, and *(b)* more streptococcic infections are detected than when fresh milk is cultured on blood agar.

In Denmark, a modified CAMP test was devised by Hovmand *(60)* and

Hauge and Ellingsen *(52)* for screening herds for *S. agalactiae* by culturing can samples, and for testing samples from individual cows. The test is made either by culturing the sample on blood agar containing thallium sulfate (1:3,000), crystal violet (1:750,000), and a suitable amount of *M. pyogenes* beta toxin, or by placing drops of milk on a plate containing this medium minus the toxin and inoculating the seeded areas with a beta toxin–producing culture of *M. pyogenes*. For the latter purpose, a stainless steel stamp with radiating spokes is used; hence, the method is called the "SUN CAMP" test. The disadvantage of this procedure is that infection with *M. pyogenes* is not detected. An advantage is that *S. agalactiae* colonies may be identified from their appearance on the original plate.

"Sterile" cultural tests on mastitis milk. Samples from quarters which show evidence of mastitis may be culturally negative for several reasons: *(a)* in severe clinical mastitis the secretion may be sterile due to the action of leucocytes and bacteriolysins, *(b)* certain strains of *S. agalactiae* are micro-aerophilic, as shown by Jepsen and Szabo *(70)*, *(c)* the quarters tested may recently have been infused with an antibiotic *(48)*, *(d)* in rare instances unusual organisms, such as the acid-fast organisms described by Tucker *(216)*, may fail to grow on the medium used, and *(e)* irritation from abusive machine-milking may increase the leucocyte count *(103)* and in extreme cases cause clinical evidence of mastitis *(11, 130, 231)*.

Factors not primarily associated with infection of the udder may, in rare instances, cause the secretion to be abnormal. Berger and Francis *(11)* found that in certain cows clots may appear in the milk at the onset of estrus. Certain diseases, such as leptospirosis, may cause the milk to be abnormal in appearance.

Comment. In general, a leucocyte count in excess of 500,000 per milliliter for milk from cows in full milk and a colony count of more than 200 per milliliter for fresh samples of fore milk are indicative of infection. Both the leucocyte count and colony count have limitations which must be taken into consideration in the routine diagnosing of udder infections. However, the two basic procedures described—cultural tests on incubated milk when combined with the leucocyte count and direct culturing of fresh fore milk—have been effective in programs for the elimination of *S. agalactiae*. In the use of cultural tests, it is essential that *(a)* contamination be reduced to a minimum by thoroughly cleaning and disinfecting the teats and teat ends, *(b)* samples be refrigerated during transit, and *(c)* the person making the tests have had experience with the method used.

CONTROL

Of the three principal etiologic forms of mastitis *(S. agalactiae*, other streptococci, and *M. pyogenes)*, *S. agalactiae* mastitis is at present the only one that can be eradicated. Until improved methods are developed for preventing other types of infection, it will be necessary to rely mainly on management practices to reduce the rate of clinical mastitis in infected udders. A combination of good management and sanitation, and treatment based on a laboratory diagnosis, is necessary for maximum reduction in the rate of mastitis in a herd.

Management. Practices which can be recommended on the basis of available information are *(a)* comfortable, dry, well-bedded stalls, *(b)* treatment of severe teat injuries by a veterinarian, *(c)* preventing exposure of the udder to chilling by draughts, cold concrete floors, and night pasturing during cold weather, *(d)* sanitation to reduce the degree and extent of exposure to infection, and *(e)* proper machine-milking. The latter involves operation of the machine at proper vacuum level and pulsation rate, use of teat cup liners that are in good condition and, of special importance, the removal of the teat cup assembly after milk flow ceases.

Sanitation. Extensive research in England *(1, 64)* on the modes of spread of *S. agalactiae* shows that the main vehicles of transmission are *(a)* udder washing cloths and udder washing water, *(b)* milking-machine teat cups, and *(c)* milkers' hands. It seems reasonable to assume that the same applies to *M. pyogenes* and other pathogenic organisms, since infected udders are a main source of infection. To avoid transfer of infection by the milkers' hands, the English workers suggest that hand-stripping be replaced by machine-stripping *(38, 64, 206, 213, 231, 233)*.

Disinfectants. Reduction of bacterial counts and prevention of udder infection should be regarded as two separate problems. High bacterial counts are due primarily to fecal and soil bacteria. These organisms are rarely the cause of mastitis. While sanitary methods commonly used may reduce bacterial counts sufficiently to meet regulations, they are not adequate in preventing the spread of infection because: *(a)* in ordinary udder washing a separate towel for each cow is not used and little attention is given to disinfecting the ends of the teats, *(b)* in dipping teat cups, the cups are not immersed for a sufficient length of time in the disinfectant solution, and the solution is not changed often enough, to maintain an active concentration of disinfectant, *(c)* after milking, milk left on the skin of the teat may provide a medium for the growth of any pathogenic bacteria that may be present, and *(d)* certain types of mastitis organisms, especially *M. pyogenes*, are not easily killed by disinfectants.

The principal chemicals used are chlorine in the form of sodium or calcium hypochlorite, quaternary ammonium compounds, preparations containing chlorhexidine, and iodine compounds. In vitro tests with these preparations indicate that, in the presence of 0.5–1% of milk, quaternary ammonium compounds are superior to chlorine against *M. pyogenes* and streptococci *(78, 209)*, chlorhexidine is equal to quaternary ammonium compounds against *M. pyogenes* and superior to the latter against streptococci and *P. aeruginosa (22, 209)*. The newer iodine compounds, called "iodophors", were reported as being superior to chlorine and quaternary ammonium compounds against *M. pyogenes* and *P. aeruginosa* by Johns *(71)*.

Sterilization of teat cups. Davidson *et al. (28)* reported that in one group of nine *S. agalactiae*–free heifers and two infected donor cows, when each animal was milked with a steam-sterilized teat cup cluster, two quarters of one cow became infected during a period of 16 wk. and, that in a similar group in which the teat cup clusters were not washed or sterilized between cows, five of the nine

heifers (36 quarters) became infected. Dipping teat cups in solutions of hypochlorite containing from 50 to 700 p.p.m. per million of chlorine has been found to be relatively ineffective in removing *M. pyogenes (53, 131, 209)*, and in reducing the bacterial counts of the liners *(131, 209)*. In the experiments of Stewart *et al. (209)* with teat cups contaminated with different organisms, a 1:5,000 solution of a quaternary ammonium compound and a 1:5,000 solution of chlorhexidine were more effective than a solution of sodium hypochlorite containing 200 p.p.m. of chlorine in removing *M. pyogenes* and streptococci; the chlorhexidine solution was more effective against *P. aeruginosa* than the two other solutions, and all of the three preparations were relatively ineffective in removing *Escherichia coli*. Chowdowski *(22)* found that dipping teat cups artificially contaminated with milk containing *S. agalactiae* in a 1% solution of CTAB[4] removed the organism, whereas a 2% solution of Chloros[5] failed to do so.

Application of disinfectants to the skin of the teat. For removal of *S. agalactiae* from the skin of the teat, Chowdowski *(22)* found that a 1% aqueous solution of CTAB, a 3% CTAB cream, and 3% iodine in either 70% alcohol or in a solution of potassium iodide (6%) were effective. Preparations found ineffective were a 2% solution of Chloros, a 4% iodine ointment, a 1% solution of liquid soap, and a 1% solution of Lysol. Oliver *et al. (134)* reported that immersing the teats of cows after their last milking in a 5% iodine tincture reduced the rate of "dry" cow infection with M. *pyogenes* but not with non–*S. agalactiae* streptococci.

No literature has been found on the value of dipping teats in an antiseptic solution after milking, as a preventive measure against infection. However, Chowdowski *(22)* suggests that teats with persistent sores be washed after each milking, first with a solution of a quaternary ammonium compound and then with water and, when dry, rubbed with penicillin cream or 3% CTAB cream.

Disinfection of udder cloths. It is generally agreed that a separate sterile cloth or paper towel should be used for each cow. If a common cloth is used and rinsed in a disinfectant solution between cows, it appears that quaternary ammonium compounds and chlorhexidine are preferable to chlorine. Chodowski *(22)* reported that a 1% solution of CTAB removed *S. agalactiae* from cloths contaminated with infected milk, and that 2% solutions of either Chloros or liquid soap failed to do so. However, the quaternary ammonium compounds are not highly effective against *P. aeruginosa* and coliform organisms, according to Stewart *et al. (209)*.

Comparison of relative effectiveness of sanitary practices. Hughes *(64)* compared the effect of different procedures in reducing the spread of *S. agalactiae*. A herd of ten *S. agalactiae*–free cows plus one donor cow (*S. agalactiae*–infected) was used. At the completion of each experiment the test animals were freed of infection by treatment. The donor cow was milked first and observations

[4] CTAB, or Cetavlon, is the trademark for a quaternary ammonium compound.

[5] Chloros is the trademark for a sodium hypochlorite preparation containing 10% of available chlorine.

were made for a period of 20–27 wk. No hand-stripping was used. In trials in which the teat cups were disinfected, two sets of teat cup clusters were used. As a set was removed from a cow it was rinsed in water and immersed in a hypochlorite solution containing 800 p.p.m. of chlorine for 4–5 min. The results were as follows: Disinfection of teat cups, sterile cloth for each cow and no disinfectant in the wash water—no established infections but *S. agalactiae* was isolated from the teats of all cows one or more times; disinfection of teat cups, separate cloth for each cow, and disinfectant in the wash water—*S. agalactiae* was isolated once from the teats of one cow; no disinfection of teat cups, sterile cloth for each cow, and disinfectant in the wash water—two cows became infected and *S. agalactiae* was isolated once from the teats of three cows. Hughes *(64)* concluded that failure to sterilize teat cups between cows resulted in transfer of *S. agalactiae* from cow to cow, but that the rate of spread was not as rapid as when a poor udder-washing technique was used.

Comment. The results presented indicate that quaternary ammonium compounds and chlorhexidine are preferable to chlorine, for use in water for washing udders and dipping teat cups for the prevention of udder infection. At best, all that can be expected from the presently available chemicals, as ordinarily used, is a reduction in the number of pathogens on the skin of the teats, milkers' hands, and teat cups. Further research is needed on the evaluation of available chemicals, including the newer iodine compounds, in preventing udder infection, and to develop more effective preparations than those now available.

Segregation. Segregation of clinically affected animals was first suggested by Frank in 1875 and later by others *(149)*. After the establishment of *S. agalactiae* as the principal cause of contagious mastitis, segregation and gradual replacement of animals infected with this organism were suggested as a control measure. In the first reports on the use of this method *(100, 152, 153)*, *S. agalactiae* infection was eliminated from several herds in from two to five years, and a herd started with first-calf heifers remained *S. agalactiae*–free during the 3-yr. period of observation *(152)*. These findings were confirmed by others *(27, 51, 82, 172, 207)*.

Application of the program on a larger scale showed that the average annual rate of new infection was about 10% for 75 segregated herds and 27% for 25 herds which were not segregated *(151)*. The elimination of *S. agalactiae* by segregation and replacement of infected cows is a long and tedious process, and use of the program is impractical in many herds.

Since the discovery of effective agents for freeing udders from *S. agalactiae*, systematic testing and treatment have largely replaced segregation. However, since a few infected cows fail to respond to treatment, the milking of treated cows after the negative ones is advisable. Purchased replacements should either be treated or segregated, tested and treated if infected, before addition to the herd.

Segregation of animals infected with organisms other than *S. agalactiae* appears to be impractical, although Schalm and Woods *(184)* recommended

the segregation of all shedders of *M. pyogenes*, since the infected udder is the principal reservoir of this organism.

Chemotherapy. S. agalactiae mastitis can be eliminated by treatment of all infected quarters, provideed the sanitary measures used are effective in preventing new and reinfection. In herds with sporadic clinical cases of mastitis due to non–*S. agalactiae* streptococci and *M. pyogenes*, treatment may be justified primarily for the purpose of alleviating clinical symptoms; however, quarters freed from infection often become reinfected *(38, 232)*. A high rate of clinical mastitis due to *S. uberis* and *M. pyogenes* indicates that some phase of management, especially poor milking technique, is involved *(30, 32, 130, 231)*, and that attempts to alleviate the condition by treatment alone will fail *(232)*.

In general, penicillin *(66, 149, 158, 192, 193, 205, 222, 231)* and chlortetracycline *(39, 139, 149, 157, 158, 196, 201)* have been found to be more effective than other antibiotics against infections with streptococci and micrococci, and streptomycin more effective than other agents against coliform infection *(34, 35, 175)*. Combinations of penicillin with Furacin® *(193)*, neomycin sulfate *(192)*, streptomycin *(39, 157, 158)*, and sulfathiazine *(201)* were not found to be more effective against *S. agalactiae* and *M. pyogenes* infections than penicillin alone.

In *S. agalactiae* infection, this organism can be eliminated from 80 to 90% of infected quarters by maintaining an effective level of penicillin in the milk for a period of from three to five days. This can be done by daily infusions of 25,000–100,000 units of penicillin in saline, or by one or two infusions (72-hr. interval) of 100,000 units in a water-in-oil emulsion or in an ointment. Increasing the dose beyond this level does not significantly increase the effectiveness of penicillin against *S. agalactiae (219, 231)*.

Intramuscular injections of large doses of penicillin have been used successfully by a few investigators. Sadek *(171)* reported that four of five cows were freed of streptococcic mastitis by three injections of 5,000 units per pound of body weight given at 24-hr. intervals. Murphy and Stuart *(122)* eliminated *S. agalactiae* from two cows by injecting a total of 42,000,000 units; 6,000,000 at the first injection and 3,000,000 units at 12-hr. intervals for six days. Tucker and Fuller *(221)* reported that several cows, involving 15 quarters with clinical *S. agalactiae* mastitis, were cured by two or three injections of 10,000,000 units.

Failure of penicillin or chlortetracycline to remove *S. agalactiae* from infected quarters is attributed to the extent and nature of the lesions, particularly clots in the milk ducts which prevent diffusion of the drug to all foci of infection *(54, 69, 72, 138, 203, 213)*. There is no evidence that failure of treatment is due to the development of penicillin-resistant strains *(6, 45, 213)*.

Streptococcic infections other than *S. agalactiae* are fairly susceptible to chlortetracycline *(157)*, penicillin *(205, 232)*, and oxytetracycline *(8)*, and the percentage of quarters reported as freed from infection, at least temporarily, ranges from 48 to 90. *S. dysgalactiae*, which is relatively uncommon in most herds, has been eradicated by treatment *(232)*; however, reinfection often follows successful treatment of *S. uberis*–infected quarters *(232)*.

M. pyogenees infections are fairly resistant to penicillin therapy. Recent infections are more likely to respond than are those of long duration *(14, 91, 158)*. The amounts of penicillin, chlortetracycline, and oxytetracycline ordinarily used have been reported as freeing from 20 to 80% of treated quarters from infection *(39, 139, 149, 157, 176, 196)*. Due to inadequate sanitation, reinfection frequently occurs *(38)*. Massive doses of penicillin (1,000,000 units), combined with streptomycin (1 g.), were reported as 70% effective by Schalm and Woods *(184)*. Failure of intramammary treatment has been attributed as due primarily to long duration of infection rather than to development of resistant strains, although some strains of *M. pyogenes* are highly resistant to one or more of the antibiotics commonly used *(3, 36, 39, 105, 185, 186)*. The histopathology of *M. pyogenes*–infected glands shows that the organisms may occur in foci which are surrounded by connective tissue which renders them inaccessible to infused antibiotics *(54)*. Sadek *(171)* reported that five of six cows with *M. pyogenes* mastitis were cleared of infection by three intramuscular injections of 5,000 units of penicillin per pound of body weight given at 24-hr. intervals.

Coliform mastitis (not common) usually responds to treatment with streptomycin. In mild chronic cases, intramammary infusions of 0.5 g. daily for four days eliminated the organism from 17 of 21 quarters treated by Schalm *(175)*. In severe, acute cases, Easterbrooks and associates *(34, 35)* found that intravenous injection of sulfamethazine (250 ml. of a 25% solution), followed by intramammary infusions of streptomycin as soon as the udder became pliable, resulted in recovery in about 50% of the animals treated, and that the remainder nearly always responded to intramuscular injections of 2–5 g. of dihydrostreptomycin daily, until 24 hr. after apparent return to normal.

Bacillary and yeast infections do not respond to penicillin therapy. In *P. aeruginosa* mastitis, Tucker *(218)* and Tucker and Johnson *(222)* have reported a recovery rate of 40% following intramammary infusion of neomycin sulfate. Prophylactic treatment of dry cows for the prevention of *C. pyogenes* mastitis was suggested by Pearson *(143, 144)*. No antibiotic has been found to be effective in the treatment of yeast mastitis.

Effect of eliminating infection. Milk production has been found to increase following the elimination of *S. agalactiae*. Edwards and Brownlee *(40)* reported that the yield of eight cows, following the elimination of *S. agalactiae*, was restored in the treated quarters in the next lactation. Edwards *(38)* observed one herd in which the average yield per cow increased from 600 gal. (during the year of treatment) to 800 gal. 5 yr. later. During this period, the percentage of cows which were retained beyond their fourth lactation increased from about 20 to 45%. Plastridge and Hale *(156)* observed a herd of 120 cows in which the initial rate of *S. agalactiae* infection was 78%. Over a 6-yr. period this organism was eliminated and the average yield per cow increased from 8,116 to 11,657 lb. The increase in yield was attributed, in part, to the culling of low-producing heifers which otherwise would have been needed to replace cows with mastitis. An illustration of the effect of eliminating *S. agalactiae* by treatment was given by Wilson *(231)*. In a herd of 20 cows, the aver-

age annual yield (gal.) per cow before, during, and 1 and 2 yr. after the elimination of this organism, was 630, 660, 970, and 1,080, respectively.

In quarters which were infected with non–*S. agalactiae* streptococci and *M. pyogenes*, and which were successfully treated, Crossman *et al. (25)* reported a recovery in milk yield during the lactation following treatment.

Comment. In the absence of a diagnostic laboratory, a reduction in clinical mastitis can be accomplished by good management and sanitation, and the use of first calf heifers to replace cows with a history of repeated attacks of clinical mastitis; however, *S. agalactiae* can not be eliminated. In areas where laboratory tests are available, these facilities should be used as a basis for treatment in the eradication of *S. agalactiae*, and as an aid in diagnosing other forms of mastitis. Any herd program for the reduction of mastitis by management, sanitation, and treatment should be based on the advice of an experienced veterinarian.

CONTROL PROGRAMS

Programs primarily for the control of *S. agalactiae* mastitis have been established in Connecticut, New York, Massachusetts, England, and Denmark.

Connecticut. Laboratory testing of milk samples on a state-wide basis was started in 1940. The samples are drawn by fieldmen employed by the State Department of Agriculture, tested at the Storrs Agricultural Experiment Station, and treatment done at the owner's expense. Individual quarter samples are collected from *S. agalactiae*–infected herds and pooled cow samples from *S. agalactiae*–free herds. The samples are refrigerated from the time of collection until delivered to the laboratory. The interval between retests is 2–3 wk. when *S. agalactiae*–infected quarters are treated and from 3 to 6 mo. for *S. agalactiae*–free herds.

At the start, segregation of *S. agalactiae*–infected animals was the basis for the control program. During 1946 to 1948 *(147, 148, 156)*, results obtained with penicillin therapy in 25 herds showed that by prompt treatment of all infected quarters, frequent retests, and disposal of a few animals that failed to respond to treatment, *S. agalactiae* could be eliminated from most herds in a relatively short time. Since 1946 *(154)*, treatment of all *S. agalactiae*–infected quarters, frequent retests until the herd is free of this organism, and sanitary measures have been recommended. In 1957, 1,191 herds (31,684 cows) were tested and of these 650 were *S. agalactiae*–free.

The time required to eliminate *S. agalactiae* has varied in different herds depending, in part, on the rate of initial infection, age of the cows, frequency of retests, whether or not cows which failed to respond to treatment were removed, addition of infected purchased replacements, and the extent of hygienic precautions against spread. Of 20 herds selected at random, the time required varied from 2 to 20 mo. and averaged 7 mo. *(158)*. The initial rate of *S. agalactiae* infection in these herds varied from 10 to 80%, and the proportion of infected quarters freed from this organism (including those reinfected) by one treatment averaged 80%.

Records on DHIA herds have shown that the average production per cow increased about 14% in 91 herds following elimination of *S. agalactiae*, and decreased about 10% in 24 herds which were negative and later became infected *(49)*.

New York State. A research and control program was started in 1946. The laboratory tests are made in five regional laboratories. Individual quarter samples of fore milk are collected by a veterinarian who, at the same time, examines the cows for clinical evidence of mastitis *(127, 142, 220)*. The samples are refrigerated during transportation. A special effort is made to correct faulty management practices, particularly improper care and use of the milking machine, preferably as a prerequisite to entrance into the testing grogram.

Progress has been made in reducing both the rate of *S. agalactiae* infection and the incidence of clinical mastitis. As shown in a recent report on 854 herds *(44)*, the percentage of *S. agalactiae*–infected cows was reduced from 22 to 8, the incidence of cows yielding an abnormal secretion from 13 to 7, and of cows with physically abnormal udders, from 22 to 12.

England. As summarized by the Technical Committee on Mastitis of the Agricultural Research Council in 1955 *(213)*, control measures have passed through the following stages: *(a)* treatment of infected quarters only, *(b)* treatment of all quarters of all infected cows, *(c)* treatment of all cows, and *(d)* treatment of all cows with disinfection of hands, udders, cows, and premises.

At the time of this report, treatment of all infected cows and use of antiseptic cream on the milkers' hands and udder were considered sufficient unless infection is widespread, when treatment of all cows may be advisable. Their recommended program consists of: *(a)* cultural examination of milk samples, *(b)* treatment of all quarters of infected and dry cows with two doses of 100,000 units of procaine penicillin in an oil base, with an interval of three days between infusions; *(c)* a retest 6 wk. after treatment and, after a herd has passed two negative tests, a test once yearly, and *(d)* removal of cows that resist two treatments. All purchased cows are regarded as infected and are treated immediately.

Their recommended sanitary measures are: *(a)* Use an approved disinfectant in proper strength, and change the solution as soon as efficiency is impaired by organic matter; *(b)* use a separate udder cloth for each cow; *(c)* apply an antiseptic cream to hands of milkers before each milking; *(d)* disinfect the teat cups before each cow; and *(e)* replace hand-stripping by machine-stripping. In herds with a high rate of teat sores, they suggest delaying treatment of udder infection until the sores are cured.

The three main ways by which herds have become reinfected are *(a)* purchase of an infected cow, *(b)* heifers, purchased or home-bred, which may calve with *S. agalactiae* infection (rare), and *(c)* hands of a milker who has come from an infected herd.

They report that *S. agalactiae* has been eradicated from hundreds of herds in one county and from large numbers of herds in various parts of Great Britain with considerable increase in milk yield.

Denmark. Following a series of "Sun CAMP" tests on can samples, tests

on individual cows are made and all infected cows are treated in all quarters with three doses of 25,000 units of sodium penicillin in an aluminum monostearate base, given at intervals of 48 hr. *(61)*. Hygienic measures used are *(a)* Once a day the skin of all teats of cows in the herd are rubbed with a penicillin cream (1,000 units per g.); *(b)* separate udder cloths saturated with a solution of sodium hypochlorite are used on each cow before milking, *(c)* during treatment, the floor of the stalls are cleaned and disinfected, and *(d)* treated cows are milked last.

Of 52 *S. agalactiae*–infected herds, 20 were freed of this infection by one treatment; in 25 herds reoccurring and new infections developed and these herds required from 3 to 7 mo. for elimination of *S. agalactiae;* and seven remained infected after 20 mo. Tests were made at intervals of about 4 mo. Failure to eradicate *S. agalactiae* from the seven herds was attributed to failure to use hygienic measures and to eliminate cows with persistent infection.

Comment. Progress in eradication of *S. agalactiae* by the use of organized control programs has been made in England, Denmark, and in the United States. Although the cultural methods used have differed, there is general agreement on the following procedures: *(a)* cultural tests on all cows, *(b)* treatment of all infected quarters, *(c)* frequent retests and treatment of infected quarters until *S. agalactiae* is eliminated, *(d)* disposal of cows that fail to respond to treatment, and *(e)* sanitation to reduce spread of infection.

Programs for the elimination of *S. agalactiae* require the full cooperation of the herd manager and his veterinarian, the director of the program, and the laboratory. The cultural tests used must approach 100% efficiency in the detection of *S. agalactiae*–infected quarters. This requires that the samples be collected carefully and refrigerated during transportation, and that the tests be made or supervised by a person with training in bacteriology plus experience with the procedures used.

ANTIBIOTICS IN MILK

Since 1948, it has been recognized that the milk from herds in which cows have been treated for mastitis by intramammary infusion may contain enough antibiotic to inhibit cheese-starter cultures. According to Welch *et al. (227)*, the cheese industry has tried to control this difficulty by insisting that their suppliers discard the milk of treated cows for three or more days following treatment, by testing milk for the presence of antibiotics, and by developing starter cultures which are resistant to penicillin.

Milk for human consumption which contains antibiotics is considered to be adulterated and a potential health hazard, since it is estimated that 10% of the human population is allergic to penicillin *(226)*. While penicillin has been found more often than other antibiotics in market milk *(228)*, by law there is no tolerance for the adulteration of milk with penicillin or other antibiotics *(228)*. In surveys of market milk made during 1955, the percentage of 474 samples found to contain penicillin (0.003 to 0.08 unit per milliliter) was 11.6 and, in surveys conducted in 1956, of a total of 1,706 samples, 5.9% contained

penicillin *(228)*. Antibiotic residues in milk are largely the result of improper use of mastitis preparations, which includes failure to conform to the instructions (required by law) on packaged preparations, to the effect that milk from treated quarters should be discarded or used for purposes other than human consumption for at least 72 hr. after the last treatment *(228)*.

Levels of antibiotics following treatment. Following intramammary infusion of quarters of lactating cows with 100,000 units of penicillin in an oily base, the units of penicillin per milliliter of secretion after 12, 24, 48, and 72 hr. have been found to vary from 3 to 156, 1 to 25, 0.0 to 3, and 0.0 to 1, respectively *(41, 56, 66, 168)*. When 300,000 units in an oily base were infused, Uvarov and Muggleton *(224)* found detectable amounts of penicillin (less than 0.5 unit) from four to eight days after treatment.

The penicillin content of the milk from 25 herds after infusion of the quarters of *S. agalactiae*–infected cows with 50,000 units of penicillin in an oily base was found by Hovmand *et al. (61)* to vary from 0.4 to 18 units (average 2.2 units) per milliliter on the first day following treatment. A total of 75 of the 193 cows in the 25 herds was treated. On the second day after treatment the average level was 0.04 unit per milliliter of herd milk.

Penicillin levels of the milk of cows following intramuscular injection of 5,000 units per pound of body weight were found to reach a maximum of 0.05 to 0.2 unit in from 5 to 12 hr. after treatment *(41, 168, 171)*, then gradually to decrease to less than 0.008 unit by the fourth day *(168)*. Similar amounts given intravenously by Edwards and Haskins *(41)* produced a level of 0.48 unit 2, 3, and 4 hr. after the injection. After 24 hr. the milk was negative.

Following intramammary infusion of 426 mg. of chlortetracycline, the concentration of the drug in micrograms per milliliter one, two, and three days after treatment, was found to be 50.4, 3.9, and 0.85 by Kanegis *et al. (74)*. Similar findings were reported by Randall *et al. (168)*; however, they found that traces of the drug persisted up to 3 wk. When 3 mg. per pound of body weight were injected intravenously, a maximum level of 1.6 μg. per milliliter of milk was found 8–12 hr. after treatment and none after three days *(168)*.

Intramammary infusion of 426 mg. of oxytetracycline resulted in the presence of detectable amounts of the drug up to five days after treatment, in trials made by Randall *et al. (168)*.

Test for antibiotics in herd milk. Tentative methods for the determination of antibiotics in milk were mimeographed in 1957 by the Food and Drug Administration, Washington, D. C. Although these methods provide an accurate estimate of the levels of different antibiotics in milk, they are relatively complex and require a minimum of 48 hr. for an analysis.

Simpler, more rapid, but less sensitive tests which may be of practical use to dairy plants have been described. The tests require from 2 to 4 hr. Several methods and the minimum concentrations of antibiotics that can be detected by their use have been reported as follows: Berridge *(12)*, 0.015 unit of penicillin per milliliter; Neal and Calbert *(128)*, 0.04 unit of penicillin, 0.3 μg. of chlortetracycline and oxytetracycline, and 4.0 μg. of streptomycin per milliliter;

Shahani and Badami *(189)*, 0.1 p.p.m. of penicillin and chlortetracycline and 0.3 p.p.m. of streptomycin and oxytetracycline, and Shipper and Petersen *(190)*, 0.05 μg. of chlortetracycline and 0.075 μg. of oxytetracycline per milliliter.

Suggestions for eliminating antibiotics in market milk. As a step in this direction, the penicillin content of single packaged doses of preparations for intramammary infusion was reduced to 100,000 units by order of the Deputy Commissioner of Food and Drugs on August 12, 1957. It seems doubtful that this measure alone will be highly effective. An educational program designed to inform dairymen of the need for withholding milk from treated quarters for a period of 72 hr. also has been proposed *(226)*. Such a program should also include information on the limitations of antibiotics in mastitis control and the need for veterinary and laboratory assistance in their use. The use of tests for antibiotics by dairy plants to eliminate antibiotics from the milk supply was suggested recently by the USDA, Federal Extension Service.

Two other possibilities have been considered: 1. The restricting of the use of antibiotics to licensed veterinarians. 2. Requiring the addition of an innocuous dye, which would color the milk for a period up to 72 hr. after infusion, to any antibiotic that is packaged for intramammary use.

It appears that an educational program is preferable to other methods and that it may be necessary to supplement the program with tests for antibiotics at the dairy plant.

CONCLUSIONS

The view that infection is the primary cause of mastitis is supported by the findings that *(a)* mastitis can be produced experimentally by injection of udders with known etiologic agents; *(b)* infection is usually associated with mastitis, and *(c)* predisposing factors contribute to mastitis mainly by increasing the chances for infection to occur or by lowering the resistance of infected quarters, thereby increasing the rate of clinical mastitis.

Predisposing factors that influence the chances for infection to occur are degree of exposure, degree of inherited resistance to infection, age, ability of the teat canal to prevent passage of microorganisms, and prolonged milking. Factors that may lower the resistance of infected quarters are chilling, excessive feeding of high protein concentrates, stage of lactation, incomplete milking, prolonged milking, and the condition and type of teat cup liner. Recently, attention has been given to the effect of the ration on mastitis. The finding that certain rations increase or decrease to some extent the ability of milk to resist acid production by *S. agalactiae* probably has little to do with invasion of the udder by pathogenic bacteria, because evidence has been presented to show that invasion of the gland is controlled largely by the characteristics of the teat canal. Further research is needed to show that the ration may be a factor in determining the extent of clinical mastitis in infected quarters.

Infection usually follows passage of pathogenic organisms through the teat canal, either by growing their way through or by suction during milking. If infection is established, inflammation results in changes in the udder secretion

and glandular tissue. These changes may or may not be associated with clinical symptoms of varying degrees of severity. Quarters infected with streptococci and *M. pyogenes* usually show mild clinical symptoms at irregular intervals, and a reduction in milk secretion with or without the appearance of clinical symptoms.

The principal organisms found in infected quarters are *S. agalactiae*, other streptococci (mostly *S. uberis*), and *M. pyogenes*. *S. agalactiae* infection is unquestionably contagious and there is some evidence that infection with *M. pyogenes* is contagious. Mastitis due to miscellaneous organisms, such as members of the coliform group, *P. aeruginosa*, *C. pyogenes*, and yeasts, is usually sporadic and is a herd problem in less than 1% of the herds surveyed in Connecticut and New York.

In diagnosis, tests that may be used in the barn are useful in estimating the extent of mastitis in a herd, and laboratory tests are essential when the control of *S. agalactiae* mastitis is attempted. In general, a leucocyte count of 1,000,000 or more per milliliter, excepting cows in early or late lactation, and a count of 200 or more per milliliter of a known pathogen, are indicative of infection. Routine laboratory procedures that have proven useful in diagnosis are *(a)* cultural tests on incubated milk when combined with a microscopic examination for the detection of streptococci and for the determination of the leucocyte count, and *(b)* cultural tests on fresh fore-milk samples from individual quarters, when the number and kinds of organisms found are used in interpreting the results. The use of any cultural method requires that skin contamination be reduced to a minimum by careful disinfection of the teat ends before drawing the samples, and that the samples be refrigerated during transit.

In control, prevention of infection is of first consideration and treatment is secondary. *S. agalactiae* can be eliminated by the use of frequent cultural tests and prompt treatment of all infected quarters, provided sanitary measures are reasonably effective in preventing new and reinfections. Programs organized for the control of *S. agalactiae* infection require the close cooperation of the dairyman and his veterinarian, the director of the program, and the laboratory. In most *S. agalactiae*–infected herds, the cost of eradicating this organism is more than compensated for by an increased milk yield, reduction in the rate of clinical mastitis, and prolonged productive life of cows in the herd. In quarters successfully treated, the increase in yield is most marked during the following lactation.

No adequate program has been devised for the elimination of udder infections with organisms other than *S. agalactiae*. This is primarily due to the ineffectiveness of present sanitary measures in eliminating pathogenic organisms, especially *M. pyogenes*, from the skin of the teat, teat cups, and milkers' hands when hand-stripping is used. A reduction in clinical mastitis due to non–*S. agalactiae* streptococci and *M. pyogenes* usually can be accomplished by improved management, especially by the use of proper milking practices. These involve operation of the milking machine at the proper vacuum level and pulsation rate, removal of the teat cups as soon as milk flow ceases, and the use of

teat cup liners kept in good condition and replaced as soon as evidence of deterioration or loss of elasticity is seen. In herds in which management and sanitation are above average, a reduction in the rate of infection with *M. pyogenes* and non–*S. agalactiae* streptococci can be accomplished by the use of periodic tests and treatment of infected quarters, and by gradually culling cows with persistent infection.

To prevent adulteration of market milk with antibiotics, dairymen should be informed of the need for discarding milk from treated quarters for a period of 72 hr. after treatment and, if this fails, the next logical procedure would seem to be the testing of herd milk for antibiotics at the dairy plant.

For more efficient application of available knowledge on mastitis control, an educational program is needed. The program should emphasize *(a)* elimination of *S. agalactiae* by the use of treatment based on laboratory tests, *(b)* reduction in the spread of infection by good sanitation, *(c)* reduction in clinical mastitis by improved management, especially the use of proper milking techniques, *(d)* that treatment of clinical cases only will not reduce the rate of udder infections, and *(e)* that veterinary assistance is needed in planning and carrying out a herd program for the reduction of mastitis.

Research is needed to improve ways of preventing udder infection, especially with *M. pyogenes* and non–*S. agalactiae*. It appears that consideration should be given to *(a)* the development of more effective bactericidal agents and methods, for removing pathogenic organisms from the skin of the teat and teat-cups, and *(b)* continued improvement in milking machines.

The ultimate goal in mastitis control should be the establishment of herds that are free from all forms of udder infection, because infection results in pathologic changes in the mammary tissue and a reduction in milk yield, with or without the occurrence of clinical symptoms.

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FACTORS INFLUENCING GROWTH AND TOXIN PRODUCTION IN CHEESE INOCULATED WITH SPORES OF *CLOSTRIDIUM BOTULINUM* TYPES A AND B. I. STUDIES WITH SURFACE-RIPENED CHEESE TYPE I [1]

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SUMMARY

Studies were conducted to determine the maximum brine concentrations that *Clostridium botulinum* Types A and B would tolerate before ceasing growth and toxin production in heated, surface-ripened cheese referred to as Type I. The flora of Type I cheese has not been reported, but consists of bacteria and yeasts. Data from four series of experiments are presented. In the three groups of trials involving moderately ripened cheese at its normal pH, cheese with pH raised, and cheese at its normal pH with added stabilizer, the maximum brine concentrations at which toxin was produced were similar for all groups. Growth and toxin production occurred in cheese with brine concentrations up to more than 8.0% when Type A spores were used; with Type B spores, the maximum brine concentrations tolerated were near 6.0%. Well-ripened cheese was a poorer medium than that moderately ripened.

Millions of jars of vacuum-packed cheese spreads have been consumed and in only one jar was *Clostridium botulinum* toxin detected. This single reported case stimulated research to study the many factors influencing growth and toxin production of *C. botulinum* in surface-ripened cheese and cheese spreads. These factors included salt, moisture, stabilizer, emulsifier, pH, and metabolites of microorganisms naturally occurring in the cheese. Salt, moisture, and pH are interrelated, and it would be difficult to study one in the absence of the others.

In general, salt in sufficient concentration inhibits the growth of *C. botulinum* *(2, 3)*. Similarly, in commercial practice, the pH of vacuum-packed cheese spreads must be kept below a certain level if bacterial activity is to be controlled during storage or display of the products.

Most cheese spreads as processed today are not sterile; consequently, any heat-resistant organisms (spores) that survive pasteurization may grow if the medium is not inhibitory. Milk, the basic ingredient in the manufacture of cheese, contains saprophytic, spore-forming anaerobic bacteria. These organisms also, including *C. botulinum*, whose natural habitat is soil, could gain access to the cheese during the processing and curing operations. The presence of sporulating anaerobic bacteria in cheese and cheese spreads increases the importance of determining the conditions for controlling these potential spoilage organisms.

The cheese studied include three of the types defined as soft surface-ripened varieties. To avoid the use of brand names, the three types of surface-ripened cheese studied will be referred to as Types I, II, and III. Experiments with

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each type will be treated in separate papers although, where pertinent, comparisons of all three types will be made. The flora of the cheese referred to in this paper as Type I contains bacteria and yeasts, but the flora of Type I cheese has not been described in detail.

Experiments with cheese without added emulsifier (as contrasted to cheese spread) will be presented and discussed in these papers. The presentation of data from several experiments in each table is necessary to establish the following basic conclusions: *(1)* that each of the three Type A *C. botulinum* cultures used produced toxin in surface-ripened cheese preparations of essentially the same brine concentrations. Any slight advantage appeared to be in favor of Strain 62A; therefore, it was used as the test culture of choice in more experiments as the research progressed; and *(2)* that usually there were differences in the maximum brine concentrations tolerated by each strain of Type A *C. botulinum*. Greater differences were observed in batches of the same type of cheese than were demonstrated between a pair or among three experimental variations (e.g., when comparing cheese at its normal pH with the same cheese with increased pH). As was expected, different batches of unheated surface-ripened cheese ripened differently under the same storage conditions. The magnitude of the differences in the ripened product were large when several similar batches of cheese were tested as media for toxin production by *C. botulinum*. In some of the tables, the data from the two experiments for a specific *C. botulinum* culture do not represent the extremes of variability in toxin production among the several batches of cheese tested. In general, experiments showing the highest brine concentrations at which toxin was produced by each strain of *C. botulinum* under the conditions studied were selected as representative ones, because they showed the greatest toxin hazard encountered for that specific experimental variable.

The purpose of these studies was to determine which factors had the greatest influence on growth and toxin production of *C. botulinum* in the three types of surface-ripened cheese. From the data obtained, recommendations can be made as to which conditions of formulation or processing are more apt to yield cheese spreads that will be free from botulism.

Although growth of *C. botulinum* spores was not specifically measured in these experiments, the phrase "growth and toxin production" will be used in the text to specify the activity of *C. botulinum*. Toxin production is evidence of growth, but this does not rule out the possibility that there may be some growth without the production of a detectable level of toxin.

MATERIALS AND METHODS

The normal salt content of surface-ripened cheese, Type I,[3] was in excess of 3%. Approximately 10% of distilled water was added to the cheese to yield a favorable brine concentration in the basic product. Part of the added moisture evaporated during the initial heating in an open pail in a water bath adjusted to 70° C. A homogenous mixture was obtained by stirring with a large spoon

[3] Type I referred to exclusively in this paper, unless otherwise indicated.

and a motor-driven stirrer. Two hundred-gram portions were accurately weighed into 400-ml. beakers. Sufficient sodium chloride was added to some of the samples to yield series having salt levels at 0.5% increments above the basic level. After making desired adjustments of the salt or pH level of the samples, six at a time were pasteurized for 10 min. in a water bath at 90° C. Immediately after pasteurization, the samples were cooled to below 60° C. Sterile distilled water was then added to each beaker until its gross weight was the same as before the heat treatment. All glassware and processing equipment were sterilized by autoclaving prior to use.

The following strains of *C. botulinum* were used in these experiments:

Strain	*Type*	*Obtained from*
T	A	Isolated in laboratory of Dept. of Microbiology, Univ. of Chicago, from an unpurified culture from cheese spread, received from the Food Research Laboratories, New York, N. Y.
62A	A	Dr. K. F. Meyer, Hooper Foundation, Univ. of Calif.
109	A	Dr. K. F. Meyer, Hooper Foundation, Univ. of Calif.
126.7	B	Department of Microbiology, Univ. of Chicago.
1KM	B	Isolated from cheese spread at Hooper Foundation, U. of Calif.
51KM	B	Isolated from cheese spread at Hooper Foundation, U. of Calif.
6B	B	Department of Microbiology, Univ. of Chicago.

Spore suspensions of *C. botulinum* were produced by inoculating 350 ml. of 5% trypticase broth in 500-ml. Erlenmeyer flasks with culture and incubating at 30° C. in a Pyrex desiccator which had been evacuated and in which anaerobiosis was maintained with sodium pyrogallate in an atmosphere of 10% CO_2. After ten days, the organisms were harvested by centrifugation for 15 min. at high speed in an International Clinical Centrifuge (angle type). The sediment was washed twice in a phosphate buffer solution at pH 7.0. The spore suspensions were heated at 85° C. for 10 min. and counts determined by making decimal dilutions and inoculating Prickett tubes of beef infusion at pH 7.2. Spore suspensions of known population were reheated for 10 min. at 85° C. immediately before being used for making appropriate dilutions and inoculating the cheese preparations with a given number of toxin-free spores. Thirty-gram portions of well-mixed cheese were weighed into sterile 25- by 150-mm. cotton-plugged culture tubes. The cheese preparation remaining in each beaker was dispensed to a screw-capped sample jar for subsequent use in determining moisture, salt, and initial pH levels. The entire 60 tubes were transferred to a desiccator jar in which an anaerobic atmosphere of 90% N_2 and 10% CO_2 was established. Incubation was at 30° C. Samples were removed for toxin and pH analyses after the number of days indicated for each experiment.

Toxin was assayed by intraperitoneal injection of white mice with 0.3-ml. portions of the supernatant fluid from centrifuged 1:5 aqueous dilutions of the cheese. The mice were observed for four days. When a test mouse died, a series of three mice was inoculated with 0.3 ml. of the toxic sample. One of the mice received in addition 0.1 ml. of Type A antitoxin and the second, 0.1 ml. of Type B

antitoxin. The third mouse was an unprotected control. Whenever a mouse injected with supernatant fluid from an uninoculated cheese died, another mouse was tested. In practically every instance the second mouse lived. In a few cases uninoculated cheese samples were accidentally contaminated with *C. botulinum* spores during processing. When this occurred, and there were unexpected deaths of mice, the presence of toxin was verified and the type determined by injections into mice protected with antitoxin. The quantity and concentration of supernatant fluid tested routinely (0.3 ml. of a 1:5 aqueous dilution of cheese) was shown by thousands of tests to be nontoxic from nonspecific protein breakdown products of cheese. Therefore, a cheese sample causing the death of a mouse unprotected by the correct type of antitoxin contained a minimum of approximately 15 m.l.d. of *C. botulinum* toxin per gram.

A Model H2 Beckman potentiometer was used for the pH determinations on undiluted cheese. Moisture analyses were made by the vacuum-drying method described by Van Slyke and Price *(4)*. Salt determinations were made according to modifications of the method described in a laboratory manual, "Methods of Analysis for the Butter Industry" *(1)*. Neither moisture nor salt levels appear among the tabular material in the tables, because comparisons of the experiments can best be accomplished if these data are first converted to brine concentrations by using the following formula:

$$\% \text{ brine} = \frac{\% \text{ salt}}{\% \text{ moisture} + \% \text{ salt}} (100).$$

In the earlier experiments, large inocula (1,000,000 per gram) of toxin-free spores were used, since difficulty was encountered in producing toxin in cheese preparations inoculated with Type B *C. botulinum* spores. Good toxin production was observed when similar preparations were inoculated with Type A cultures; therefore, the inoculum for many subsequent trials was lowered to 10,000 spores per gram. One Type B *C. botulinum* strain yielded toxic samples at the lower brine concentrations tested; hence, the smaller inoculum was also used with this culture. In a few experiments the inoculum was 100 spores per gram.

RESULTS

Mildly ripened cheese Type I usually has a pH of approximately 5.7 to 5.8. The pH rises slowly upon further aging in the refrigerator, and when pH 6.2 is attained the cakes of cheese have a uniform, soft consistency. Thus, this stage was chosen as the division between what subsequently will be termed "moderately ripened" and "well ripened" cheese. Some batches had a pH higher than 6.2 while still retaining the body characteristics of a relatively fresh cheese. The higher pH was especially noticeable in cakes ripened in dealers' showcases in commercial, parchment-lined foil wrappers, as compared to bulk lots ripened in a polyethylene bag encased in a fiberboard container stored at 2–4° C.

Several trials were conducted with strains of three Type A and one Type B *C. botulinum,* to determine the maximum brine concentration (salt level) these

strains would tolerate either in moderately or in well ripened Type I cheese before ceasing growth and toxin production.

Studies on moderately ripened, heated cheese at its normal pH. Data on two trials with four cultures in moderately ripened cheese are included (Table 1).

In general, with the three Type A *C. botulinum* cultures tested, growth and toxin production occurred in cheese preparations having brine concentrations ranging from 6.95 to 8.00% (salt levels of 4.30–5.05%). In all but one of the experiments, toxin was present at the highest salt level by the end of 30 days of incubation at 30° C. In some instances, toxin was present at the lower brine concentrations after three days of incubation; in others, the first toxic samples appeared at seven days. In all trials, the level of salt necessary to inhibit growth and toxin production was higher as the incubation period was prolonged (usually up to 30 days). With the Type B culture the brine concentrations necessary to inhibit growth and toxin production were lower; the levels that yielded toxin samples after 90 days of incubation were in the range between 5.55–5.85% (salt levels of 3.35–3.70%). The toxin production by Type B cultures was slower than by Type A; therefore, the first analysis was conducted after seven days of incubation.

The initial and final pH readings of the cheese in each experiment are given. With the Type A strains, the pH dropped steadily during incubation, varying from 5.2 to 5.7 by the end of 60 days. With the Type B culture, no definite pH pattern was established, but the terminal pH at 90 days was usually above the initial reading.

Four previous experiments with three other Type B cultures inoculated into moderately ripened cheese produced toxin in only one. In these trials, the inoculum was 1,000,000 spores per gram. In one experiment with Strain 1KM, no toxin was produced in cheese with a brine concentration of 4.55% and an

TABLE 1

Toxin production in moderately ripened, heated, surface-ripened cheese (Type I) inoculated with toxin-free spores of Clostridium botulinum

(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
1	T	A	6.2	5.4	5.40	7.00	7.95	7.95	7.95
2[b]	T	A	6.1	5.2	6.00	6.95	6.95	6.95	6.95
3	62A	A	5.9	5.5	4.90	6.50	7.15	8.00	8.00
4	62A	A	6.0	5.5	(5.45)	6.25	6.95	6.95	7.75
5	109	A	5.9	5.5	(5.95)	5.95	6.80	7.40	7.40
6	109	A	5.9	5.7	5.40	6.15	6.80	7.70	7.70
					7 days	14 days	30 days	60 days	90 days
7	126.7	B	5.8	6.6	(5.55)	(5.55)	5.55	5.55	5.55
8	126.7	B	5.8	6.5	5.15	5.15	5.15	5.15	5.85

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.

[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.

initial pH of 6.1; in a second trial, with fresher cheese, with an initial pH of 5.8, toxin was produced at a brine concentration of 5.35%. With Culture 51KM in cheese with an initial pH of 6.2, no toxin was present at a brine concentration of 5.15%. Similarly, no toxin was produced by Culture 6B at a brine concentration of 4.95% in cheese with an initial pH of 6.1.

Studies on well-ripened, heated cheese at its normal pH. Data on experiments with three Type A cultures in well-ripened cheese are given (Table 2). The number of these trials conducted with well-ripened cheese were not as numerous as those with moderately ripened. Toxin production by Type B cultures was not active, even in moderately ripened cheese, and the number of trials with Type B *C. botulinum* in well-ripened cheese was limited. A comparison of the data of Tables 1 and 2 revealed poorer growth quality in well-ripened cheese. The cheese used in Experiment 13 was extremely well-ripened. Exceptions to the general trend were also observed. The well-ripened cheese used in Experiment 10 was a satisfactory medium for growth and toxin production of Type A *C. botulinum* spores. Similarly, the cheese used in Experiment 15 supported growth of Type B spores at a brine level as high as that observed with comparable samples of fresher cheese (Table 1).

Studies on moderately ripened, heated cheese with pH increased with NaOH solution. C. botulinum normally grows and produces toxin more readily in a medium with a pH near neutrality; therefore, raising the pH of surface-ripened cheese to a higher level might possibly improve it as a medium for growth and toxin production. Data from several trials in moderately ripened cheese preparations, with pH adjusted upward by adding concentrated NaOH solution, are given (Table 3). Growth and toxin production of the three Type A strains tested occurred in adjusted pH preparations having brine concentrations up to the range 7.00–8.30% (salt levels of from 4.40 to 5.15%). Essentially, this was

TABLE 2

Toxin production in well-ripened, heated, surface-ripened cheese (Type I) inoculated with toxin-free spores of Clostridium botulinum

(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
9	T	A	6.4	6.4	(4.95)	(4.95)	(4.95)	(4.95)	(4.95)
10[b]	T	A	6.5	5.6	(5.25)	5.25	7.10	7.10	7.10
11	62A	A	6.4	5.7	(4.85)	4.85	5.55	5.75	5.55
12	62A	A	6.6	6.4	(5.10)	(5.10)	(5.10)	(5.10)	(5.10)
13	109	A	7.1	6.9	(5.60)	(5.60)	(5.60)	(5.60)	(5.60)
14[c]	109	A	6.3	5.2	(5.30)	5.30	(5.30)	6.25	5.30
					7 days	14 days	30 days	60 days	90 days
15	126.7	B	6.3	5.5	(5.90)	(5.90)	(5.90)	5.90	5.90

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.

[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.

[c] The test samples in Experiment 14 received an inoculum of 100 spores per gram.

the same range as observed for the cheese preparations at their normal pH level. The general pattern of an increase in the level of brine tolerated as the incubation period progressed also was noted. In samples in which toxin was produced, the terminal pH levels in both the normal and adjusted pH samples were essentially the same. From a comparison of the data (Tables 1 and 3), it was concluded that adjustment of the pH of moderately ripened cheese to a level closer to neutrality did not significantly improve it as a growth medium for *C. botulinum* and, in some instances, poorer toxin production was observed. In one experiment, in which Strain 126.7 (Type B) was inoculated into moderately ripened cheese adjusted to a higher pH with NaOH solution, results similar to those for comparable cheese at its normal pH were obtained. Experiments in which other Type B cultures (1KM, 51KM, or 6B) were added to moderately ripened cheese after pH adjustment were all negative for toxin production at the lowest salt levels tested. In the experiments with the latter three strains, the inoculum was 1,000,000 spores per gram.

Studies on moderately ripened cheese, heated with stabilizer added and at its normal pH. Stabilizer is routinely added to many cheese spreads to prevent brine water from separating from the product. The highest salt levels at which growth and toxin production by *C. botulinum* occurred were determined in surface-ripened cheese with nothing added except water and salt, and in some cases NaOH solution. Stabilizer (sterile locust bean gum) was added to many cheese preparations tested. In the earlier trials, 0.8% of stabilizer was used, but after encountering difficulty in handling some of the fresher cheese preparations with this level of stabilizer, the amount added was lowered to 0.4%. In most of the later experiments, the routine addition was 0.2%. The data (Table 4) from experiments on moderately ripened cheese containing locust bean gum showed that, in most instances, with strains both of Type A and Type B tested,

TABLE 3

Toxin production in moderately ripened, heated, surface-ripened cheese (Type I) with pH increased before inoculation with toxin-free spores of Clostridium botulinum

(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
16[b]	T	A	6.6	5.4	Not tested	6.30	7.00	7.00	7.00
17	T	A	7.2	5.7	5.35	6.15	6.15	7.00	7.75
18	62A	A	6.7	5.4	(6.40)	7.15	7.15	7.15	8.00
19	62A	A	6.6	5.5	(5.35)	6.05	7.65	7.65	7.65
20	109	A	6.6	5.2	5.35	6.70	7.55	7.55	7.55
21	109	A	6.6	5.5	(7.55)	(7.55)	7.55	8.30	8.30
					7 days	14 days	30 days	60 days	90 days
22	126.7	B	6.5	5.7	5.00	5.60	5.60	5.60	(5.00)

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.

[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.

TABLE 4

Toxin production in moderately ripened, heated, surface-ripened cheese (Type I) with added stabilizer and inoculated with toxin-free spores of Clostridium botulinum

(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
23[e]	T	A	5.9	5.3	(5.75)	6.50	7.20	8.00	8.00
24[c, f]	T	A	6.1	5.2	5.00	6.55	6.55	7.35	7.35
25[b, d]	62A	A	6.1	5.2	(5.20)	5.75	6.90	6.90	6.90
26	62A	A	5.9	5.1	(5.80)	(5.80)	6.55	6.55	7.30
27[b]	109	A	5.9	5.2	(6.00)	6.00	6.00	6.85	7.65
28	109	A	5.8	4.9	(5.25)	5.25	6.05	6.05	6.05
					7 days	14 days	30 days	60 days	90 days
29	126.7	B	5.8	4.9	(5.65)	(5.65)	(5.65)	(5.65)	(5.65)
30	126.7	B	5.8	5.7	(5.30)	5.30	5.30	5.70	5.30

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.

[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.

[c] The test samples in Experiment 24 received an inoculum of 100 spores per gram.

[d] 0.8% stabilizer added to cheese in Experiments 25, 29, and 30.

[e] 0.4% stabilizer added to cheese in Experiments 23, 26, 27, and 28.

[f] 0.2% stabilizer added to cheese in Experiment 24.

growth and toxin production occurred at salt levels comparable to those observed in similar trials with moderately ripened cheese without added stabilizer. The addition of stabilizer produced a uniform dispersion of the free moisture. The brine concentrations at which growth and toxin production occurred with Type A cultures varied from 6.05 to 8.00% (salt levels of 3.70–5.05%). Toxin was produced almost as well by Strain 126.7 (Type B) when stabilizer was present as was observed without stabilizer (see also Table 1).

DISCUSSION

Although Type B *C. botulinum* toxin was found in only one jar of commercially packed cheese spread, it was observed in laboratory studies that Type A *C. botulinum* spores grew and produced toxin in cheese preparations having higher brine concentrations than when Type B spores were used as the inocula. Consequently, most experiments were conducted with inocula of Type A spores, because this produced the most severe test in a cheese medium of the safety of any specific combination of factors (e.g., moisture, salt, pH, etc.). In addition, the environmental conditions for *C. botulinum* were improved by incubating the inoculated cheese samples in desiccators which were made anaerobic by exhausting the air and absorbing the O_2 with sodium pyrogallate. Approximately 10% of an atmosphere of CO_2 was admitted to the desiccator before incubation.

The fact that *C. botulinum* toxin was produced in many instances at sodium chloride brine concentrations up to more than 8.0% should not convey the idea that natural surface-ripened cheese is a potentially good medium for toxin pro-

duction by *C. botulinum*. In the first place, toxin was produced by *C. botulinum* in the presence of high brine concentrations only when the cheese had been heat-treated to destroy the thermolabile microflora. Unpasteurized surface-ripened cheese was a poor medium for growth of *C. botulinum*; the natural microorganisms overgrew the *C. botulinum* spores unless they were added in large numbers. Secondly, it was shown that the addition of disodium phosphate as an emulsifier in preparing cheese spreads contributed significantly to inhibiting growth and toxin production of *C. botulinum* in heat-treated cheese preparations.

With both Type A and B cultures of *C. botulinum* used in these trials, toxin was produced at approximately the same salt levels, regardless of whether the inoculum was 10,000 or 1,000,000 spores per gram. Similarly, in experiments to be reported, it will be observed that 100 spores per gram oftentimes produced toxin at essentially the same brine concentrations as with the larger inocula. Toxin production was delayed in cheese with a high brine concentration, but was formed in a shorter time if the inoculum of *C. botulinum* spores was increased.

In moderately ripened, heated cheese, toxin was produced at approximately the same brine concentrations by the three Type A strains tested. The adjustment of the pH upward with NaOH solution to levels closer to pH 7.0 did not improve these preparations as media for growth and toxin production by the test cultures. With both the normal and adjusted pH samples, the maximum salt levels tolerated were in the range 4.30–5.15%. This range is above the salt level acceptable in a soft cheese product; therefore, a palatable concentration of salt can not be depended upon as the sole inhibitor of growth and toxin production by *C. botulinum* in ripened, heated cheese.

The poor toxin production by *C. botulinum* in well-ripened cheese was probably due to breakdown products that were inhibitory. One possibility considered was that certain fatty acids were involved. However, experiments indicated that fatty acids were not contributing as much of the inhibition in well-ripened cheese as was anticipated. Similarly, the addition of small percentages (less than 50%) of well-ripened to a moderately ripened cheese did not make the blend a significantly poor medium for growth and toxin production.

In the earlier trials, when 0.8% stabilizer was added to moderately ripened cheese, the results indicated that growth and toxin production usually did not occur at as high brine levels. This difference may be attributed to the finer dispersion of the salt brine and the lessened tendency for separation during incubation when stabilizer was used. In later experiments, with lower levels of stabilizer, most brine concentrations permitting growth and toxin production by *C. botulinum* were comparable to those where no stabilizer was present.

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FACTORS INFLUENCING GROWTH AND TOXIN PRODUCTION IN CHEESE INOCULATED WITH SPORES OF *CLOSTRIDIUM BOTULINUM* TYPES A AND B. II. STUDIES WITH SURFACE-RIPENED CHEESE TYPE II [1]

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SUMMARY

Studies were conducted to determine the maximum brine concentrations *Clostridium botulinum* Types A and B would tolerate before ceasing growth and toxin production in heated surface mold-ripened cheese designated Type II. Data from four series of experiments are presented. In the three groups of trials involving this cheese with initial pH levels above 5.7, the maximum brine concentrations at which toxin was produced varied widely. Growth and toxin production by the three strains of *C. botulinum* Type A tested was better in cheese without stabilizer than with stabilizer added. In two comparable groups with stabilizer, the maximum brine concentrations associated with growth and toxin production by both Type A and Type B *C. botulinum* were higher in the adjusted pH cheese preparations than in those at their normal pH level. Type II cheese with added stabilizer and initial pH levels of 5.7 or below was a poorer medium than similar preparations with pH levels above 5.7, but was an equally good medium for growth and toxin production of *C. botulinum* when the pH was adjusted upward with NaOH solution. Growth and toxin production occurred in cheese with brine concentrations up to more than 7.0% when Type A spores were used; with Type B spores, the maximum brine concentrations tolerated were near 6.0%.

Surface-ripened cheese (Type II[3]) is a mold-ripened cheese; therefore, some of the breakdown products of the ripening process are different from those produced by bacteria during surface ripening. To determine if this cheese was satisfactory for growth and toxin production for *Clostridium botulinum*, experiments were conducted using it as the growth medium.

MATERIALS AND METHODS

The normal salt content of Type II cheese was in excess of 3%. The moisture level of this cheese was lower than that observed with surface-ripened cheese Type I; therefore, approximately 15%, rather than 10%, distilled water was added to yield a favorable brine concentration in the basic product. The other materials and methods used in these experiments were the same as in the studies reported for Type I *(1)*.

RESULTS

After observing a rise in pH on ripening Type I cheese at 2–4° C., it was assumed that a similar trend might occur in Type II cheese. However, a study of the initial pH levels of Type II cheese revealed two interesting facts. First, the pH of factory-fresh Type II cheese varied more widely than the pH of fresh

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[3] Type II referred to exclusively in this paper, unless otherwise stated.

Type I cheese. Several shipments of Type II cheese with an initial pH between 5.8 and 5.9 were received, and some had a pH below 5.7. Secondly, after refrigerating Type II cheese for several days or weeks, it can not be anticipated if the pH will rise or fall. Therefore, pH was not a satisfactory guide as to whether Type II cheese was "moderately ripened" or "well ripened." In several experiments, all samples were negative for toxin. The initial pH of the Type II cheese used in these experiments appeared to be inhibitory to growth and toxin production of *C. botulinum*. Therefore, these experiments are reported in two categories: namely, *(1)* where the initial pH of the cheese was above 5.7, and *(2)* where it was 5.7 or lower.

Studies on heated Type II cheese with pH levels above 5.7. Experiments were conducted using heated cheese with initial pH levels above 5.7 as the growth medium. Data on trials with strains of three Type A and one Type B *C. botulinum* are presented (Table 1). In general, with each of the Type A *C. botulinum* cultures tested, growth and toxin production occurred in cheese preparations with brine concentrations up to the range 5.00–6.60% (salt levels of 3.00–4.00%). In one instance, toxin was present at the lowest brine concentration after three days of incubation at 30° C.; in others, the first toxic samples appeared at seven or 14 days. In most of the trials, the level of salt necessary to inhibit growth and toxin production was higher as the incubation period was prolonged. With the Type B strain (126.7) the maximum brine concentration necessary to inhibit growth and toxin production was lower. The brine level that yielded toxic samples was 5.95% (salt content of 3.60%).

The initial and final pH readings of the cheese in each experiment are given. With the Type A strains, the pH decreased during incubation within the range 5.1–5.4 by the end of 60 days. With Type B culture, no definite pH pattern was established, but the pH level at 90 days was below the initial reading.

Studies on heated Type II cheese with pH levels above 5.7 with added

TABLE 1

Toxin production in heated, surface-ripened cheese (Type II) with pH levels above 5.7 and inoculated with toxin-free spores of Clostridium botulinum

(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
1	T	A	5.8	5.3	(5.35)	(5.35)	5.35	5.70	5.70
2[b, c]	T	A	5.9	5.4	5.25	5.25	6.15	6.55	6.55
3[b]	62A	A	5.9	5.3	(5.25)	5.25	6.05	6.60	6.60
4	62A	A	5.8	5.4	(5.00)	(5.00)	5.00	5.00	(5.00)
5[b]	109	A	5.8	5.1	(5.10)	5.10	5.10	5.90	6.55
					7 days	14 days	30 days	60 days	90 days
6	126.7	B	5.9	5.7	(5.10)	5.10	5.10	5.95	5.95

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.

[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.

[c] The test samples in Experiment 2 received an inoculum of 100 spores per gram.

stabilizer. After determining the highest salt levels at which growth and toxin production by *C. botulinum* would occur in cheese preparations with only moisture and salt added, stabilizer was added to many of the subsequent cheese preparations tested. In two of the earlier trials, 0.8% of stabilizer was used, later 0.4% and, finally, the routine addition was standardized at 0.2%. Data from experiments on cheese with initial pH levels above 5.7 containing sterile locust bean gum are given (Table 2). With strains of Type A *C. botulinum* tested, growth and toxin production did not occur at as high salt levels as in similar trials with Type II cheese without added stabilizer (see also Table 1). The brine concentrations at which growth and toxin production occurred with the Type A cultures varied from 5.35–6.45% (salt levels of 3.05–3.80%). In one experiment, with the lowest brine concentration, toxin was not produced at the end of all time intervals tested. Toxin was produced with an inoculum of 100 spores per gram at brine concentrations within the range observed with a higher inoculum (10,000 spores per gram). Toxin was produced as well by Strain 126.7 when stabilizer was present as without stabilizer. Decreases in pH levels occurred in toxic samples of Type II cheese similar to those in Type I cheese inoculated with *C. botulinum.*

Studies on heated Type II cheese with added stabilizer and with pH levels above 5.7 increased with NaOH solution. C. botulinum grows and produces toxin more readily in a medium with a pH near neutrality; therefore, raising the pH of the cheese to a higher level might improve it as a medium for growth and toxin production. Data from several trials in cheese preparations adjusted upward by the addition of concentrated NaOH solution are given (Table 3). With the three Type A cultures tested, growth and toxin production occurred in adjusted pH cheese preparations having brine concentrations from as high

TABLE 2

Toxin production in heated, surface-ripened cheese (Type II) with added stabilizer and initial pH levels above 5.7 and inoculated with toxin-free spores of Clostridium botulinum

(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
7[b]	T	A	5.8	5.1	(5.35)	(5.35)	(5.35)	5.35	(5.35)
8[d, e]	T	A	6.1	5.6	(6.00)	(6.00)	(6.00)	(6.00)	6.00
9	62A	A	5.9	5.3	(5.30)	(5.30)	5.30	5.30	6.15
10[c]	62A	A	5.8	5.2	(5.25)	(5.25)	(5.25)	(5.25)	(5.25)
11	109	A	5.9	5.1	(5.05)	(5.05)	5.85	5.85	5.85
12	109	A	6.2	5.3	(5.40)	5.40	6.45	6.45	6.45
					7 days	14 days	30 days	60 days	90 days
13	126.7	B	5.9	5.1	(5.20)	(5.20)	(5.20)	6.05	6.05

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.

[b] 0.8% stabilizer added to cheese in Experiments 7 and 9.

[c] 0.4% stabilizer added to cheese in Experiments 10, 11, and 13.

[d] 0.2% stabilizer added to cheese in Experiment 8 and 12.

[e] The test samples in Experiments 8, 11, and 12 received an inoculum of 100 spores per gram.

as 6.25–7.40% (salt levels of 3.50–4.35%). This range was above the brine concentrations observed at their normal pH level. The general pattern of an increase in the level of brine tolerated as the incubation period progressed was also noted. Smaller differences were observed in one experiment with Type B strain (126.7). In samples in which toxin was produced, the terminal pH levels in both the normal and adjusted samples were in most instances essentially the same. From these data it was concluded that the adjustment of Type II cheese with initial pH above 5.7, to a level closer to neutrality, improved it as a growth medium for *C. botulinum*.

TABLE 3

Toxin production in heated, surface-ripened cheese (Type II) with added stabilizer and initial pH levels above 5.7 but increased before inoculation with toxin-free spores of Clostridium botulinum

(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
14[c]	T	A	6.2	5.4	(5.55)	(5.55)	6.25	6.25	6.25
15[b, d, f]	T	A	6.3	5.3	(5.15)	6.00	6.85	6.85	6.85
16[b]	62A	A	6.3	5.4	(5.30)	5.30	5.30	7.00	7.00
17	62A	A	6.4	5.1	(5.35)	6.05	6.70	6.05	6.70
18[b, e]	109	A	7.0	5.6	(5.60)	(5.60)	6.50	7.40	7.40
					7 days	14 days	30 days	60 days	90 days
19	126.7	B	6.4	5.8	(5.50)	(5.50)	5.50	6.25	5.50

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.

[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.

[c] 0.8% stabilizer added to cheese in Experiments 14 and 16.

[d] 0.4% stabilizer added to cheese in Experiments 15, 17, and 19.

[e] 0.2% stabilizer added to cheese in Experiment 18.

[f] The test samples in Experiments 15 and 18 received an inoculum of 100 spores per gram.

Studies on heated Type II cheese with added stabilizer and with pH levels of 5.7 or below. Data from several trials with cheese with initial pH levels of 5.7 or below are given (Table 4). In only one experiment in which the cheese was inoculated with Type A spores was *C. botulinum* toxin produced; here, the cheese had a brine concentration of 5.55% (salt content of 3.15%). Growth and toxin production by spores of *C. botulinum* did not occur at as high brine concentrations in cheese preparations having pH 5.7 or below as in similar experiments with cheese with initial pH levels above 5.7. In fact, in some experiments, it appeared that the pH of the cheese and not the salt was the principal inhibitory agent. In the one experiment where Type B spores were used, the lowest brine concentration prevented toxin production during all time intervals tested.

DISCUSSION

In heated Type II cheese preparations with pH levels above 5.7, before inoculation with Type A strains of *C. botulinum* spores, the maximum salt concentrations in which toxin production occurred were in the range 3.00–4.00%.

TABLE 4

Toxin production in heated, surface-ripened cheese (Type II) with added stabilizer and initial pH levels of 5.7 or below inoculated with toxin-free spores of Clostridium botulinum
(Inoculum—100 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
20[b]	T	A	5.6	5.1	(5.65)	(5.65)	(5.65)	(5.65)	(5.65)
21[c]	T	A	5.5	5.8	(5.35)	(5.35)	(5.35)	(5.35)	(5.35)
22	62A	A	5.6	5.3	(4.80)	(4.80)	(4.80)	(4.80)	(4.80)
23	62A	A	5.6	5.5	(5.20)	(5.20)	(5.20)	(5.20)	(5.20)
24[d]	109	A	5.6	5.0	(5.55)	(5.55)	(5.55)	(5.55)	5.55
25	109	A	5.6	5.4	(5.95)	(5.95)	(5.95)	(5.95)	(5.95)
					7 days	14 days	30 days	60 days	90 days
26	126.7	B	5.6	5.3	(4.95)	(4.95)	(4.95)	(4.95)	(4.95)

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.
[b] 0.4% stabilizer added to cheese in Experiments 20, 22, 24, 25, and 26.
[c] 0.2% stabilizer added to cheese in Experiments 21 and 23.
[d] The test samples in Experiment 24 received an inoculum of 10,000 spores per gram.

With one Type B strain, toxic samples were obtained where the salt level of the cheese was 3.60%. In similar cheese preparations with pH levels below 5.7 before spore inoculation, the maximum salt level tolerated was 3.15%. In some of these latter experiments it was apparent that the pH of the cheese and not its salt content was the principal inhibitory agent.

In similar cheese preparations, where the pH was raised by the addition of NaOH solution, growth and toxin production of *C. botulinum* occurred at salt concentrations of 2.80–4.70%, whether the initial pH of the cheese was above or below 5.7. Data are not presented from experiments in which cheese with initial pH levels of 5.7 or below was adjusted to higher pH levels with NaOH solution, but it was observed that growth and toxin production were attained at a higher brine concentration in all trials at the adjusted pH levels. The improvement of these cheese preparations as growth media was greater than in cheese having initial pH levels above 5.7. Cheese with initial pH levels of 5.7 or below was usually inhibitory to growth and toxin production by *C. botulinum* and, apparently, was not a good medum until the pH level was raised to 6.0 or higher. This was in contrast to Type I cheese, where good toxin production was observed in preparations having pH levels ranging from 5.7 to 6.0. From the experiments in which Type II cheese was tested at both natural and adjusted pH levels, it appeared that the adjustment of the pH to a level closer to neutrality significantly improved it as a growth medium for *C. botulinum* and increased the quantity of salt required for inhibition.

Furthermore, from a comparison of the data, it was concluded that Type I cheese was a better medium for growth and toxin production of *C. botulinum* than was Type II.

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FACTORS INFLUENCING GROWTH AND TOXIN PRODUCTION IN CHEESE INOCULATED WITH SPORES OF *CLOSTRIDIUM BOTULINUM* TYPES A AND B. III. STUDIES WITH SURFACE-RIPENED CHEESE TYPE III [1]

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SUMMARY

Experiments were conducted to determine the maximum brine concentrations required to inhibit growth and toxin production of *Clostridium botulinum* Type A in heated, surface-ripened cheese designated as Type III cheese. This cheese is ripened primarily by bacteria. Data from three groups of trials are presented. With inocula of Type A spores, growth and toxin production occurred in cheese with brine concentrations up to 6.50%. In two groups with moderately ripened cheese at the normal pH, the maximum brine concentrations permitting growth and toxin production were in the same range in cheese with stabilizer as in similar cheese with no stabilizer. Cheese preparations with increased pH were poorer media for *C. botulinum* than was the cheese with normal pH.

Major observations in comparable experiments with surface-ripened cheese designated Types I, II, and III are discussed.

Surface-ripened cheese designated Type III [3] in this paper is ripened primarily by bacteria. Experiments were conducted to determine growth and toxin production of *Clostridium botulinum* in this cheese. Poor growth response was observed with this cheese inoculated with spores of *C. botulinum*. These experiments were limited to the use of Type A spores of *C. botulinum* as inocula, since Type A *C. botulinum* was observed to grow under less favorable conditions.

MATERIALS AND METHODS

The normal salt content of this Type III cheese was in excess of 3%. The moisture level was lower than that observed with Types I or II; therefore, approximately 20% distilled water was added to yield a favorable brine concentration in the basic product. The other materials and methods used were the same as were described for studies on Type I *(1)*.

RESULTS

There was more variation in the initial pH levels of the batches of this cheese than in Types I or II. The Type III cheese was from two manufacturers. Each brand of cheese had a different pH range. The term "moderately ripened" here refers to cheese that was ripened sufficiently so that it could be melted into a homogenous mixture without the use of an emulsifier. The age of the cheese varied from 5 to 13 wk., using a blend of two or three lots of different ages.

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[1] These studies were supported by a grant from the National Cheese Institute.

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[3] Type III referred to exclusively in this paper, unless otherwise stated.

Studies on moderately ripened, heated Type III cheese at its normal pH. Data with three Type A *C. botulinum* cultures are given (Table 1). In four of the five experiments, growth and toxin production occurred in cheese preparations having brine concentrations from 5.50 to 6.00% (salt levels of 2.90–3.70%). In the other trial, the lowest salt level tested was inhibitory to *C. botulinum*. In two instances, toxin was present after seven days of incubation at 30° C. In other experiments, the first toxic samples appeared at 14 or 30 days. In most of the trials, the level of salt necessary to inhibit growth and toxin production was higher as the incubation period was prolonged.

The initial pH of the cheese varied from a low of 5.9 to a high of 7.2. Decreases in pH levels occurred in toxic samples in the same way as observed for Types I and II which were inoculated with Type A *C. botulinum*, although the pH levels at 60 days usually were not as low.

Studies on moderately ripened, heated Type III cheese with added stabilizer at its normal pH. Data on cheese with added stabilizer are given (Table 2). In four of five experiments in which cheese preparations were inoculated with Type A *C. botulinum* spores, growth and toxin production occurred at brine concentrations in the range 5.50–6.50% (salt levels of 3.05–3.95%). In one experiment, the lowest salt level tested was inhibitory to *C. botulinum*. There were no differences in the brine concentrations allowing growth and toxin production in the presence of stabilizer, when compared to similar cheese with no stabilizer added (Table 1). The initial pH of the cheese varied from a low of 6.0 to a high of 7.1, and the ripening period was within the stated range for Type III cheese without stabilizer.

Studies on moderately ripened, heated Type III cheese with added stabilizer and pH increased with NaOH solution. Data from several trials with surface-ripened cheese preparations adjusted upward by the addition of concentrated NaOH solution are given (Table 3). In three of the experiments, growth and toxin production occurred in raised pH preparations having brine concentrations in the range 4.40–6.05% (salt levels of 2.65–3.60%). In two trials, the

TABLE 1

Toxin production in moderately ripened, heated, surface-ripened cheese (Type III) inoculated with toxin-free spores of Clostridium botulinum
(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
1	T	A	6.6	6.1	(5.50)	(5.50)	(5.50)	5.50	5.50
2[b]	T	A	6.1	5.4	(4.35)	5.15	6.00	6.00	6.00
3[b]	62A	A	6.2	5.8	(4.25)	(4.25)	4.90	5.70	5.70
4[b,c]	62A	A	7.2	6.6	(3.85)	3.85	3.85	4.75	5.55
5	109	A	5.9	6.1	(5.80)	(5.80)	(5.80)	(5.80)	(5.80)

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.

[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.

[c] The test samples in Experiment 4 received an inoculum of 100 spores per gram.

TABLE 2

Toxin production in moderately ripened, heated, surface-ripened cheese (Type III) with added stabilizer inoculated with toxin-free spores of Clostridium botulinum
(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
6[b,e]	T	A	6.1	5.5	(4.65)	4.65	5.35	6.30	6.30
7	T	A	6.3	5.3	(5.50)	(5.50)	(5.50)	5.50	5.50
8[b,e,f]	62A	A	7.1	6.5	(4.25)	(4.25)	4.25	4.95	5.90
9[b]	62A	A	6.7	5.6	(4.55)	4.55	6.50	6.50	6.50
10[d]	109	A	6.0	5.8	(6.35)	(6.35)	(6.35)	(6.35)	(6.35)

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.
[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.
[c] The test samples in Experiments 8 and 9 received an inoculum of 100 spores per gram.
[d] 0.8% stabilizer added to cheese in Experiment 10.
[e] 0.4% stabilizer added to cheese in Experiments 6 and 7.
[f] 0.2% stabilizer added to cheese in Experiments 8 and 9.

TABLE 3

Toxin production in moderately ripened, heated, surface-ripened cheese (Type III) with added stabilizer and with pH increased before inoculation with toxin-free spores of Clostridium botulinum
(Inoculum—10,000 spores per gram)

Experiment No.	Culture	Type	Initial pH	Final pH	Brine concentrations (%) in which toxin was produced after incubation at 30° C. for[a]				
					3 days	7 days	14 days	30 days	60 days
11[c]	T	A	6.8	5.6	(4.70)	4.70	4.70	4.70	5.55
12	T	A	7.0	6.7	(5.65)	(5.65)	(5.65)	(5.65)	(5.65)
13[b]	62A	A	6.8	6.1	(4.50)	4.50	5.30	5.30	6.05
14[c,f]	62A	A	7.8	5.7	(4.40)	(4.40)	(4.40)	4.40	4.40
15[d]	109	A	6.4	6.3	(6.00)	(6.00)	(6.00)	(6.00)	(6.00)

[a] Toxin was not detected in samples where brine percentage is in parentheses; brine concentration given in parentheses indicates the lowest salt level tested.
[b] The highest brine concentration listed was the highest one tested in this particular experiment; hence, there was a possibility that toxin would have been produced in a higher concentration.
[c] The test samples in Experiment 14 received an inoculum of 100 spores per gram.
[d] 0.8% stabilizer added to cheese in Experiment 15.
[e] 0.4% stabilizer added to cheese in Experiments 11, 12, and 13.
[f] 0.2% stabilizer added to cheese in Experiment 14.

lowest salt level tested was inhibitory to *C. botulinum*. This range was below the brine concentrations observed for the cheese preparations at their normal pH level (Table 2). More salt as measured by increased brine concentration was less inhibitory for the growth of *C. botulinum* as the incubation period was extended. In toxic samples the pH levels at 60 days in both the normal and adjusted samples were essentially the same. From these data it was concluded that raising the pH of this cheese with concentrated NaOH solution generally made it a poorer medium for *C. botulinum*. This was the reverse of conditions usually observed with cheese Types I and II. In some instances the initial pH of the adjusted Type III cheese was 7.0 or higher which, apparently, is above the optimum pH range for *C. botulinum* in a cheese medium. Furthermore, the

data on the experiments (Tables 1, 2, and 3) indicated that this Type III cheese was a poorer medium for growth and toxin production of *C. botulinum* than was either Type I or Type II *(1, 2)*.

DISCUSSION

Toxin was produced in heated cheese preparations, both with and without stabilizer added, by three Type A strains of *C. botulinum* at salt levels of 2.90–3.95%. When the pH was adjusted by the addition of NaOH solution, growth and toxin production occurred at salt concentrations of 2.65–3.60%. The maximum salt levels favoring toxin production were lower than observed in Type I or Type II cheese, heat-processed and stored in a similar manner *(1, 2)*. The higher brine concentrations needed to inhibit the activity of *C. botulinum* were consistent in preparations made with Type I cheese.

Type A spores of *C. botulinum* grew and produced toxin at a higher brine concentration than Type B spores in two of the three types of surface-ripened cheese. Because of the generally poor growth response with *C. botulinum* in Type III cheese, only Type A spores were used.

C. botulinum toxin was produced at approximately the same salt levels with an inoculum of 100 or 10,000 spores per gram in all three types of cheese. In many instances, the rise to the maximum salt level tolerated was not as rapid, i.e., a greater percentage of the cheese preparations was negative for toxin at the three- and seven-day analyses.

There were differences in the effect of raising the pH of the three types of surface-ripened cheese. When Type I cheese preparations were adjusted upward from pH levels within the range 5.8–6.2, they were not improved as media for the test cultures. In contrast, when Type II cheese preparations were adjusted upward from pH levels within the range 5.8–6.2, there was a marked increase in the maximum brine concentrations permitting growth and toxin production. Furthermore, Type II cheese preparations with initial pH levels within the range 5.5–5.7 were usually completely inhibitory to the growth of *C. botulinum*. It appeared that the pH of the cheese and not its salt content was the principal inhibitory agent. These preparations were significantly improved as growth media by adjusting their pH levels to 6.0 or higher. A third trend was observed when the pH of Type III cheese was adjusted upward with concentrated NaOH solution; raising the pH of the cheese usually made it a poorer medium for *C. botulinum*. In some instances, the initial pH of the adjusted Type III cheese was 7.0 or higher, which apparently is above the optimum pH range for *C. botulinum* in this cheese.

With the three types of surface-ripened cheese tested, the maximum salt levels tolerated by *C. botulinum* spores were, in some experiments, in a range above the salt level acceptable in a soft cheese product; therefore, a palatable concentration of salt can not be depended upon as the sole inhibitor of growth and toxin production in heated surface-ripened cheese.

Except for a few earlier trials, in which 0.8% stabilizer (sterile locust bean gum) was added, there were no indications that the addition of stabilizer changed

the maximum brine concentration tolerated by *C. botulinum* in any of the three types of surface-ripened cheese. The inhibitory levels of brine concentration affecting the growth and toxin production of *C. botulinum* in cheese were unchanged by 0.4 or 0.2% stabilizer.

Of the three surface-ripened cheeses tested, Type I was the best medium for growth and toxin production of *C. botulinum* Types A and B; Type II cheese, second; Type III, poorest. The reason that one cheese was a poorer medium than the other two was not specifically determined. Studies on this problem are in progress.

The experiments reported in the three papers in this series were tabulated and discussed in detail to establish several basic concepts regarding the behavior of surface-ripened cheese Types I, II, and III, when they were heat-processed and subsequently inoculated with toxin-free spores of Type A or Type B *C. botulinum*. Whenever sufficient data were available, the results of two experiments with each test culture were tabulated under each experimental variable. These data were presented to show that there were usually as great or greater differences in the individual batches of cheese as media for toxin production than were observed among comparable experiments, in which different strains of Type A *C. botulinum* spores were used as the inoculum. The importance of the initial pH of the cheese as a factor contributing to the potential suitability of a particular batch of cheese as a medium for toxin production was also emphasized. This factor was important in the studies with Type II cheese. The conditions for growth and toxin production by *C. botulinum* were made as favorable as possible in these initial studies to determine the maximum brine concentrations in which toxin would be produced by Types A and B spores. In all experiments reported here, the cheese preparations were heat-processed at 90° C. for 10 min. prior to being inoculated with heat-shocked *C. botulinum* spores. To further improve conditions for growth and toxin production, the inoculated cheese samples were incubated in a desiccator in an atmosphere containing approximately 10% CO_2. These optimum conditions yielded data which served as a basis for evaluating other factors which make natural, unheated, surface-ripened cheese and heat-processed cheese spreads relatively poor media for growth and toxin production by *C. botulinum*. In experiments to be reported subsequently, data will be presented comparing toxin production in unheated cheese with that observed in pasteurized and autoclaved cheese. Other experiments will show the influence of using disodium phosphate or sodium citrate emulsifiers on growth and toxin production by *C. botulinum* in surface-ripened cheese. Finally, data will be presented on inoculated cheese spreads prepared in small-scale pilot-plant equipment, and stored in commercial-type glass jars.

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IDENTITY OF ESTERS PRODUCED IN MILK CULTURES OF *PSEUDOMONAS FRAGI* [1]

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SUMMARY

Isovalerate and acetate esters were the principal esters detected in the steam distillate of a milk culture of *Pseudomonas fragi*. In an analysis of the acids present in similar culture distillates, isovaleric acid was the most prominent acid detected which was not present in the uninoculated control milk. Ethanol was the only alcohol detected in the cultured milk and was not found in the control milk. Supplementation of sterile milk with ethanol, prior to culturing, stimulated rapid production of esters by the organism. In such cases, the isovalerate ester was most prominent. Four of a panel of six judges were able to detect as little as 0.01 p.p.m. of ethyl isovalerate in a series of milk samples, and all described the aroma of milk dilutions of this ester as very similar to that of *P. fragi* milk cultures. Evidence is presented which indicates that the production of esters responsible for the fruity aroma in milk cultures is markedly affected by the previous nutrition and/or incubation temperature of *P. fragi* cells used as inoculum. The role of leucine as a probable substrate in the production of the isovalerate ester by *P. fragi* is discussed.

The fruity aroma defect occurring in dairy products is caused primarily by the psychrophile, *Pseudomonas fragi*. This organism was first isolated by Eichholz *(5)* from milk possessing a strawberry-like odor. Hussong *et al. (6)* likened the odor of their cultures to that of the flower of the May Apple. The literature reveals little of the nature of the compounds responsible for the ester-like aroma of such cultures, although Omelianski *(8)* maintained that isoamyl isovalerate was responsible. This contention was not supported by experimental data or references to previous investigations. He also claimed that the aroma production was dependent upon the presence of nitrogenous organic matter.

In the course of a study of the nutrition of *P. fragi*, Pereira and Morgan *(9)* observed that the typical fruity aroma was produced when the organism was grown in a basal salts medium containing leucine, alanine, or glutamic acid as a carbon source, or in a salts glucose medium containing threonine as a nitrogen source.

The present study was undertaken to identify the volatile compounds responsible for the characteristic ester-like aroma produced in milk cultures of *P. fragi*.

EXPERIMENTAL PROCEDURE

P. fragi Strain 1 CH_7, isolated from fruity cottage cheese, was used throughout this study. Twenty-liter batches of pasteurized-homogenized milk distributed

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in 500-ml. quantities in one-liter flasks were autoclaved for 10 min. at 10 p.s.i. Amino acid supplements when included were added to the milk prior to autoclaving at a rate of 1 g. of the DL form per liter. Supplements with liquid compounds were made at a rate of 1 ml. per liter prior to autoclaving, except that methanol and ethanol were added after the heat treatment.

The milks prepared in this manner were inoculated with 1 ml. per liter of a 24-hr. nutrient broth culture incubated at room temperature, or a 14-day milk culture incubated at 8° C. The inoculated milks were incubated at 8° C. and examined daily for aroma development. After aromas had developed, the 20-liter quantities of culture were steam-distilled and 50 ml. of distillate collected per liter of culture. When the distillates were to be analyzed for acids, the milk cultures were adjusted to pH 1.0 with dilute H_2SO_4 immediately prior to distillation. The distillates were extracted with 25 ml. of ether in a continuous liquid-liquid extractor for 24 hr.

Detection of esters. Ether extracts of the culture distillates were examined for esters by the method of Cantarelli *(2)*. Two milliliters of freshly prepared alkaline hydroxylamine solution (prepared by mixing equal volumes of methanolic solutions of 5% hydroxylamine hydrochloride and 3 *N* NaOH and filtering to remove NaCl) was added to the ether extract. After 1 hr. at room temperature the mixture was acidified with 0.15 ml. of glacial acetic acid. The ether was then evaporated by blowing a stream of air over the solution. Twenty to forty microliters of the residual hydroxamate solutions was spotted on Whatman No. 1 paper and developed by the descending method, with the upper phase of a *n*-amyl alcohol-glacial acetic acid-water mixture (74:19:50), after equilibration of the paper in an atmosphere of the aqueous phase overnight. After development, the chromatogram was dried at room temperature and sprayed with a saturated solution of ferric chloride in *n*-butanol. The ferric hydroxamates appeared immediately as violet spots on a yellow background. When a separation of valerohydroxamate from isovalerohydroxamate was desired, a *n*-octanol-formic acid-water solvent (75:25:75) was employed *(11)*.

Detection of acids. Acids present in the steam distillates from acidified milk cultures and a 20-liter lot of uninoculated control milk were converted to their methyl esters by the use of diazomethane as described by Bergmann and Segal *(1)*. An ether solution of diazomethane, freshly prepared by the method of DeBoer and Backer *(4)*, was added to the ether extracts of the distillates until a persistent yellow color developed. The mixture was allowed to stand at room temperature for 20 min., after which time a few drops of 0.4 *N* HCl in ethanol were added to destroy the excess reagent. The resulting methyl esters of the volatile acids were then converted to hydroxamic acids and chromatographed in the manner previously described for the analysis of esters.

Detection of alcohols. The 3,5–dinitrobenzoates of the volatile alcohols present in the milk cultures and controls were prepared by adding 0.5 g. of 3,5–dinitrobenzoyl chloride to 25 ml. of the ether extract of the steam distillates and refluxing for 10 min. *(3)*. The ether was evaporated and the residue dissolved in 2–4 ml. of chloroform. Chromatographic identification of the 3,5–dinitroben-

zoates was achieved by the method of Meigh *(7)*, except that the Whatman No. 1 paper to be used was saturated with a methanolic solution (20 mg. per ml.) of Rhodamine B instead of Rhodamine 6 GBN. 500, then dried at 100° C. Ten to thirty microliters of the chloroform solution of the derivatives was spotted on the papers and descending chromatograms developed. The upper phase of a methanol-heptane solvent (1:2) was used as the developing solvent after equilibration of the paper overnight in an atmosphere of the lower methanol-rich phase. Spots containing microgram quantities of authentic 3,5–dinitrobenzoates of nine alcohols ranging from methanol to 3-methyl-1-butanol were not visible in daylight, but were revealed under ultraviolet light (3660A) as blue areas on a fluorescent yellow background.

Reference derivatives employed in the chromatographic identification of the volatile compounds from milk cultures were prepared from commercially available compounds by the procedures described above.

RESULTS

Paper chromatography of the hydroxamic acid derivatives of the acids obtained from milk cultures of *P. fragi* revealed the presence of propionic and isovaleric acids after 2 wk. of incubation at 8° C., in addition to the formic, acetic, and butyric acids detectable in the distillates from the uninoculated control milk. Paper chromatography of the 3,5–dinitrobenzoates of the volatile alcohols in similar cultures indicated the presence of ethanol only. No alcohols were detected in the control milk and ethanol appeared in the cultures only after an incubation period of 4 wk. Although the components of esters were detected in this series of cultures after extended incubation, esters were never present in detectable quantities, even though faint fruity aromas were detected over the cultures at various times during the incubation period.

In light of the results of the above experiment, and because of the apparent similarity between the typical fruity aroma produced in dairy products and the odor of ethyl isovalerate in low concentration, an attempt was made to stimulate ester formation by *P. fragi* by supplementation of the milk medium. Since leucine would appear to be the most likely source of isovaleric acid necessary for the synthesis of the ester, leucine was added to the milk medium (1 g. per liter). This addition did not stimulate the aroma production during extended incubation. Because it appeared that ethanol might be the limiting reactant in the esterification process, ethanol (1 ml. per liter) was added to the leucine-supplemented milk medium. A strong characteristic aroma was detected over cultures containing added ethanol and leucine after eight days of incubation. Subsequent chromatography of the hydroxamic acid derivatives of the esters from steam distillates of these cultures revealed spots running parallel with the hydroxamates of formic, acetic, propionic, and isovaleric acids. The hydroxamate of butyric acid was not detected. Judging from the intensities of the spots on the chromatograms, the hydroxamate of isovaleric acid was the major component followed by those of acetic, formic, and propionic acid. Since ethanol was the only alcohol present in these cultures, it is reasonable to assume that the esters detected as hydroxamates were ethyl esters.

In order to determine whether other amino acids would stimulate aroma production in the presence of added ethanol or methanol, additions of leucine, isoleucine, and valine were tested. Although methanol had no effect in these cultures, a strong characteristic aroma was detected over all cultures to which ethanol had been added with or without added amino acids.

To determine whether esters were being formed by a simple chemical esterification, or whether the esterification was actually mediated by cells of *P. fragi*, ethanol was added to sterile homogenized milk which contained isovaleric or isobutyric acid. These mixtures were incubated at 8° C. and examined daily for aroma. At the end of 14 days no fruity aroma was apparent over any of the test mixtures. Distillates of these mixtures contained no esters, as determined by paper chromatography of hydroxamic acids. These results indicate that the esterification reaction does not occur spontaneously, and is mediated by *P. fragi*.

In all the trials reported thus far, detectable amounts of esters were never produced in milk cultures in the absence of added ethanol. The inoculum in these trials was from 24-hr. nutrient broth cultures incubated at room temperature. In an attempt to increase the aroma production of the organism in unsupplemented milk cultures, a 20-liter batch of milk was cooled to the 8° C. incubation temperature and inoculated with a milk culture of the organism which had been incubated at 8° C. for 14 days. It was believed that the cells in such inoculum would be conditioned in respect to age, nutrition, and temperature of growth to be more nearly like those which find their way into dairy products during processing and which often produce a strong fruity aroma. Milk cultured in this manner developed a typical fruity aroma in 14 days and was steam-distilled on the 18th day. Paper chromatography of the hydroxamic acids formed in the ether extract of the distillate revealed spots which ran with known isovalerohydroxamate, propionhydroxamate, and acetohydroxamate. The isovalerohydroxamate and acetohydroxamate spots were of about equal intensity; whereas, the propionhydroxamate spot was barely visible.

A series of milk samples containing known amounts of ethyl isovalerate was presented to a panel of judges to determine whether additions of this compound simulates the typical fruity aroma produced by *P. fragi* in dairy products. Four of the six judges were able to detect as little as 0.01 p.p.m. of added ethyl isovalerate and all described the aroma of low concentrations of the ester as typically fruity.

DISCUSSION

The fruity aroma defect which may develop in various dairy products as the result of the metabolism of the psychrophilic contaminant, *P. fragi*, appears to be due to the formation of a group of simple esters. Since ethanol was the only free alcohol detected in milk cultures, and isovalerate ester production was stimulated by supplementation of the milk medium with ethanol, it is quite likely that the major component of the fruity aroma produced by this organism is ethyl isovalerate. There is little in the present report which indicates any specificity limitation in the esterification mechanism. While it is possible that the organism may be capable of esterifying higher alcohols as suggested by Omelianski

(8), no higher alcohol precursors of such esters were detected in culture distillates. Since ester formation by bacteria is a rather rare phenomenon, further investigation of this mechanism is warranted.

Although *P. fragi* produces ethanol on extended incubation in sterilized milk, it appears likely that its production is limiting in the esterification mechanism. This organism's ability to convert leucine to isovaleric acid when grown in a salts medium containing this amino acid as a sole source of both carbon and nitrogen *(10)*, and its ability to produce the typical fruity aroma in a similar medium in which leucine served only as a carbon source *(9)*, indicate that leucine in milk probably serves as a precursor of isovaleric acid. Since isovaleric acid was readily detected in milk cultures of the organism, it is unlikely that the availability of leucine in milk is a limiting factor in the esterification reaction.

The fact that esters were not detected in the milk cultures of *P. fragi* until they were inoculated with cells grown in milk at 8° C., instead of cells grown in nutrient broth at 20° C., suggests that the enzyme systems responsible for either the production of ethanol or the actual esterification process are favored by continued low-temperature cultivation of the organism in a milk medium.

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ISOLATION AND IDENTIFICATION OF ACIDIC AND NEUTRAL CARBONYL COMPOUNDS IN DIFFERENT VARIETIES OF CHEESE [1, 2]

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SUMMARY

By use of paper chromatographic procedures, hydrazone derivatives of acidic and neutral carbonyl compounds were isolated and identified. The identity of these compounds was established by the following methods: (1) comparison of R_f values with those of known compounds and R_f values given in the literature; (2) decarboxylation of beta-keto acids and chromatographic analysis of the resulting neutral carbonyl compounds along with known compounds, and (3) the light absorption of both the known and unknown compounds.

In the four individual cheeses studied, from four to 11 keto acids were found in each cheese and from two to eight neutral carbonyl compounds were present in the different cheeses. Keto acids identified were: oxalacetic, oxalsuccinic, glyoxylic, pyruvic, alpha-ketoglutaric, acetoacetic, alpha-acetolactic, beta-keto-isocaproic, beta-ketocaprylic, beta-ketocapric, alpha-ketobutyric, alpha-ketoisovaleric, alpha-ketoisocaproic, and alpha-ketophenylpyruvic. Neutral carbonyl compounds were: acetaldehyde, acetone, pentanone-2, diacetyl, acetylmethylcarbinol, and C-5, C-7 aldehydes or ketones.

During the past ten years, the emphasis in cheese-ripening studies has changed from an estimation of gross chemical changes to the identification and quantitative analysis of individual chemical compounds formed during ripening. Attention has been given to individual amino acids and fatty acids. These products, which may be termed primary *(6)*, have been shown to be important in characterizing cheeses and may be important also as precursors of additional products essential in ripening and flavor development. Several instances concerning changes in these groups of compounds can be cited. For example, the formation of tyramine from tyrosine has been studied in some detail in Cheddar cheese *(3)*. Also, the methyl ketones derived from fatty acids have been related to the flavor of Blue cheese *(10)*. Recent experimental evidence on a variety of metabolic pathways utilized by various microorganisms which may be present in cheese, has suggested the possible presence of many compounds in cheese in addition to those known at the present time. Attention needs to be directed to

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[2] A report of work done under contract with the U. S. Department of Agriculture and authorized by the Research and Marketing Act of 1946. The contract is being supervised by the Eastern Utilization Research and Development Division of the Agricultural Research Service.

the identification of products which may be termed secondary *(6)*, that may accumulate during the cheese-ripening process, and to determining the significance of such compounds in cheese-flavor development.

Very little attention has been given to enzyme systems such as amino acids transaminases, deaminases, or fatty acid oxidases in cheese-ripening studies, or to the products which might accumulate as a result of such enzyme action. This study was directed toward the isolation and identification of the acidic and neutral carbonyl compounds in cheese which might be present as a result of such enzymatic activities.

EXPERIMENTAL PROCEDURES

A survey of various chromatographic procedures available for the analyses of carbonyl compounds resulted in the selection of the paper chromatographic procedures of El-Hawary and Thompson *(4)* for acidic carbonyl compounds and the method of Huelin *(7)* for neutral carbonyl compounds. The method of preparing the sample for reaction with 2,4–dinitrophenylhydrazine was derived experimentally. The method described below is one which resulted in the minimum destruction of some of the more labile carbonyl products, and was used in all of the experiments reported.

Preparation of 2,4–dinitrophenylhydrazones of carbonyl compounds. A water extract of cheese was prepared by a procedure similar to that used by Sommer and Harper *(13)*. The use of heat was eliminated or kept at a minimum to prevent excess destruction of the carbonyl compounds. The methods for the preparation of the cheese extract and the reaction with 2,4-dinitrophenylhydrazine are outlined as follows:

1. *Sample preparation.* From 1 to 20 g. of freshly ground cheese were placed in a Waring Blendor, 50–100 ml. of distilled water at 25° C. were added, and the cheese then was mixed into a homogenous mass for 5 min. at high speed.

2. *Deproteinization.* After chilling to 4° C., to prevent destruction of any beta-keto acids, 5–10 ml. of 10% sodium tungstate was added, the mixture agitated for a few minutes, then acidified to pH 4.0 with 0.67 *N* sulfuric acid. The solid material was removed by centrifugation for 10 min. at 2,000 r.p.m. in an International Centrifuge (No. 1). The supernatant was filtered by suction through a Whatman No. 1 filter paper, dampened and dusted with washed silicic acid, and covered with another damp filter paper while suction was being applied. The sediment layer was added to the filter and washed several times with a total 20 to 30 ml. of distilled water at 25° C.

3. *Reaction with 2,4–dinitrophenylhydrazine.* The clear filtrate was transferred to an Erlenmeyer flask and a slight excess of 2,4–dinitrophenylhydrazine added. The amount needed was determined experimentally by adding 2–3 ml. of reagent, then testing after 20 min. to determine if an excess of reagent was present. A drop of reagent-reaction mixture was placed on a porcelain test plate and 1 ml. of *N* sodium hydroxide was added. The development of a black color, which faded away rapidly, was an indication of an excess of the reagent. For quantitative measurement, this drop was returned to the reaction mixture

after acidifying. The solution was reacted at room temperature for 60 min., then chilled to 4° C. before extracting the hydrazones.

4. *Extraction with ether.* The solution was transferred to a separatory funnel and extracted with carbonyl-free ether until all of the 2,4–dinitrophenylhydrazones were removed. If an emulsion formed, the layers were separated by centrifugation at 2,000 r.p.m. for 5–10 min.

5. *Removal of ether and drying of hydrazones.* The ether extract was transferred to a beaker (not over three-fourths full) and evaporated at room temperature or in a water bath at about 35° C. until about 10 ml. of solution remained. The residual ether was then removed by drying for 18–24 hr. at room temperature under vacuum in a desiccator with calcium chloride and potassium hydroxide.

6. *Separation of acidic and neutral carbonyl compounds.* A minimum amount of *N* ammonium hydroxide was added to the residue, using only the amount necessary to bring the carbonyl derivatives into solution. The neutral carbonyl compounds were then extracted from the alkaline solution with about 50 times as much carbonyl-free chloroform as alkaline solution. The two layers were separated by centrifugation at 2,000 r.p.m. for 10 min.

7. *Preparation of hydrazones for chromatographic separation. (a)* The ammonium layer, which contained the acidic compounds, was removed with a suction pipette; *(b)* the chloroform layer was evaporated and the dried neutral carbonyls were separated from the unreacted 2,4–dinitrophenylhydrazine by dissolving the residue in a small quantity of heptane-benzene (1:1 v/v) and passing through a packed column of anhydrous magnesium sulfate *(7)*. Elution with this solvent was continued until all phenylhydrazones were removed. The solvent was evaporated to dryness at room temperature.

Chromatographic analyses. Paper chromatographic procedures were utilized as outlined:

1. *Acidic carbonyl compounds.* A measured amount of the alkaline solution was placed on Whatman 3-MM filter paper along a line 2.0 cm. from the bottom of the paper. The bands or spots were allowed to dry. Several additions could be applied if the hydrazone concentration was low. After the bands were dried, the papers were placed in a rectangular glass chamber over a glass rod and immediately chromatographed for 20–24 hr. The solvent ascended 20 cm. and descended about 20 cm. The solvent was butanol −0.5 *N* ammonia-ethanol (70-20-10 v/v) as used by El-Hawary and Thompson *(4)*.

2. *Neutral carbonyl.* The method of Huelin *(7)* was utilized for paper chromatographic analysis of the neutral carbonyl compounds. About 0.02 ml. of the neutral carbonyls were spotted 2.0 cm. above the lower edge of Whatman No. 1 or 3-MM filter paper. It was found that equilibration of the chromatogram for 12–18 hr. in the chamber containing the immobile phase (methanol-rich layer) was absolutely essential. Incomplete equilibration resulted in markedly different R_f values. After equilibration, the heptane-rich layer was added to a glass dish in the trough which contained the filter paper. The separation was completed in 6–8 hr.

EXPERIMENTAL RESULTS

The first phase of this study was directed toward the isolation and identification of the carbonyl compounds in cheese. Except where otherwise stated, the reaction, extraction, and chromatographic procedures outlined previously were used.

Qualitative identification of known acidic carbonyl compounds. For this study, all commercially available keto acids were obtained and used for identification purposes. Hydrazones of 20 compounds were prepared as outlined in the procedure and analyzed with unknown compounds obtained from cheese. Most of the work reported in the literature on keto acids used R_f values to identify the compounds. Limited information of spectroscopic analyses was available for these compounds *(2, 4, 9)*. The methods found best suited for identification were: R_f values, color of the 2,4–dinitrophenylhydrazones, and neutral carbonyl compounds.

Before beginning analyses for keto acids all reagents were tested for carbonyl compounds. No reaction occurred when only distilled water was used to carry through the complete extraction procedure, and if 2,4,–dinitrophenylhydrazine was present in the chromatogram it moved near the solvent front with some streaking. A spot was formed with an R_f value of 0.3 if alkaline 2,4–dinitrophenylhydrazine was evaporated to dryness on a steam bath and then chromatographed.

The keto acids analyzed, the color on paper, color in 10% alkali, R_f values, and their light absorption characteristics are shown (Table 1). For these compounds, for which R_f values were available from the literature, the results agreed very closely. Seven compounds were studied that had not been previously investigated by other workers. Several compounds which had not been previously reported to form hydrazone isomers were found in several isomeric forms. Phenylpyruvic and hydrophenylpyruvic acids formed two hydrazone isomers, and mesoxylic acid formed three components, on chromatographic separation. Isomers of these compounds were always found to be present even immediately after reaction with 2,4–dinitrophenylhydrazine. As noted by other investigators*(2, 12)*, pyruvic acid hydrazone formed isomers only after aging. Most commercial keto acids, with the exception of pyruvic acid and alpha-ketoglutaric acid, contained some other keto compounds. These impurities could be differentiated from isomers by eluting all spots and rechromatographing. If isomers were present, they should continue to give spots for all components when rechromatographed.

The color of the hydrazone compounds in 10% alkali (as shown in Table 1) was very specific in grouping the compounds into classes. As noted in this work and the work of other investigators *(2, 3)*, compounds like the 2,4–dinitrophenylhydrazones of beta-keto acids give a light- to dark-brown color on paper.

The light absorption characteristics of the various hydrazone derivatives of alpha-keto acids were shown to be a reliable procedure for the definite identification when used in conjunction with R_f values. Absorption maxima of four keto acid 2,4–dinitrophenylhydrazones—oxalacetic acid, alpha-ketoglutaric acid,

TABLE 1

Characteristics for identification of acidic carbonyl compounds

Keto acid[b]	Color on paper	Color in 10% alkali	R_f values	R_f literature	Characteristic of peak maximum
			(R_f)	*(R_f)*	*(m)*
Oxalsuccinic	Dark yellow	Orange	0.08–0.10	0.80–0.10	388 Broad
Alpha-ketoglutaric	Yellow	Orange	0.18–0.20	0.12–0.19	432 Broad
Oxalacetic	Brownish-yellow	Brown	0.24–0.25	0.19–0.29	450 Medium
Mesoxylic	Yellow	Light chocolate	0.34	0.35–0.36	Decomposes
Glyoxylic (1)	Reddish-yellow	Reddish-pink	0.28–0.34	0.34	450 Very sharp
Pyruvic (1)	Yellow c	Chocolate	0.44–0.48	0.41–0.44	445 Sharp
Glyoxylic (2)	Reddish-yellow	Salmon	0.50–0.52	.....	400 Very sharp
Pyruvic (2)	Yellow	Dark yellow	0.58–0.62	0.58–0.62	400 Sharp
Alpha-acetolactic[a]	Brownish-yellow	Brown	0.64	.....	Decomposes
Beta-mercaptopyruvic	Brownish-yellow	Dark grey	0.40	.....	465 Sharp
Alpha-ketobutyric	Yellow	Yellow	0.66	0.68	428 Medium
Alpha-ketoisovaleric	Light yellow	Yellow	0.73–0.74	0.74	430 Broad
Alpha-ketovaleric	Light yellow	Yellow	0.74	0.74	430 Broad
Hydroxypyruvic	Yellow	Salmon	0.40	.....	430 Broad
Methylgamma-thio-alpha-ketobutyric	Brownish-yellow	Dark grey	0.64	0.64	
Acetoacetic	Brownish-yellow	Light brown	0.54	0.48–0.60	460 Medium
Beta-ketovaleric	Brownish-yellow	Brown	0.58	.....	Decomposes
Beta-ketocaproic	Brownish-yellow	Brown	0.60–0.62	.....	Decomposes
Beta-ketocaprylic	Brownish-yellow	Brown	0.70–0.71	.....	Decomposes
P-hydroxyphenyl-pyruvic (1)	Light yellow	Dark yellow	0.70	0.72	440 Broad
Beta-ketocapric	Brownish-yellow	Brown	0.76–0.78	.....	Decomposes
B-hydroxyphenyl-pyruvic (2)	Light yellow	Dark yellow	0.76	.....	
Phenylpyruvic (1)	Light yellow	Dark yellow	0.74	0.76	440 Broad
Alpha-ketocaproic	Light yellow	Yellow	0.80	0.80	430 Very broad
Alpha-ketoisocaproic	Light yellow	Yellow	0.80	0.80–0.82	430 Very broad
Phenylpyruvic (2)	Light yellow	Dark yellow	0.80	.....	

[a] Isolated from cheese, not commercially available.

[b] Numbers following keto acids designate isomers.

and two isomeric pyruvic acid derivatives—have been reported previously in the literature *(4, 9)*. These absorption maxima agreed with those in Table 1. The absorption curves in the visible range, as measured in a scanning spectrophotometer, are shown for representative keto acids (see Figures 1, 2, 3, and 4).

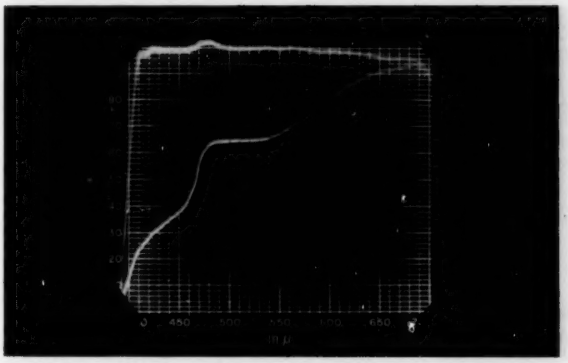

FIG. 1. Visual light absorption pattern of oxalsuccinic acid phenylhydrazone acid in 10% sodium hydroxide.

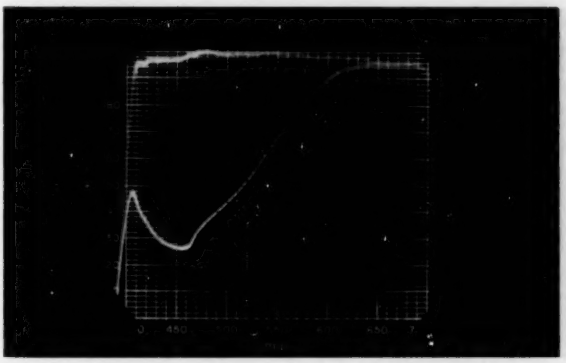

FIG. 2. Visual light absorption pattern of glyoxylic acid phenylhydrazone in 10% sodium hydroxide.

The top curve represents a 10% sodium hydroxide reference control, and the bottom curve represents the hydrazone in 10% sodium hydroxide *(2, 7)*. It can be seen that the compounds can be readily differentiated on the basis of their absorption characteristics.

Since beta-keto acid hydrazones partially decomposed during the extraction procedure for light absorption analysis, these acids were identified by decarboxylating the derivative and chromatographing the resulting neutral hydrazone compound.

The characteristics of 2,4-dinitrophenylhydrazone derivatives of different neutral carbonyl compounds are shown (Table 2). This chromatographic method

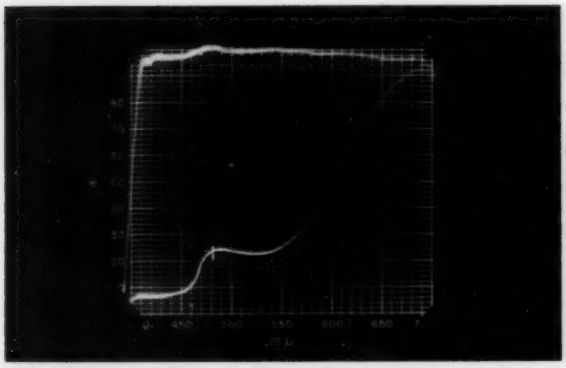

FIG. 3. Visual light absorption pattern of alpha-ketoglutaric acid phenylhydrazone in 10% sodium hydroxide.

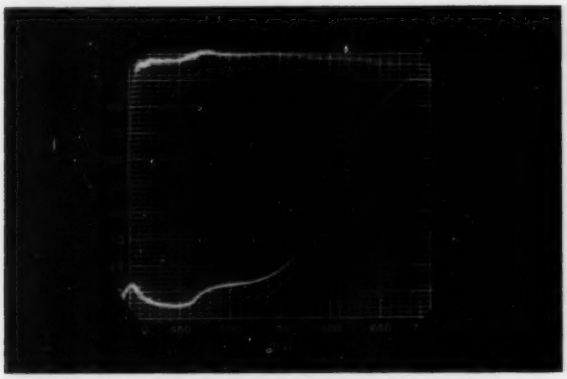

FIG. 4. Visual light absorption pattern of pyruvic acid phenylhydrazone in 10% sodium hydroxide.

TABLE 2

Characteristics of neutral carbonyl compounds

Hydrazone compound	Color on paper	Color in alkali	R_f values	Maximum absorption
				(m)
Glyoxal	Reddish	Purple	0	380
Formaldehyde	Yellow	Brown	0.28	349
Diacetyl	Orange	Red	0.28	394
Acetaldehyde	Yellow	Brown	0.42	358
Acrolein	Red	Red	0.35	366
Crotonaldehyde	Red-yellow	Red-brown	0.38	372
Acetylmethylcarbinol	Yellow	Orange	0.48	350
Acetone[a]	Yellow	Brown	0.55	358
Propional[a]	Orange	Brown	0.55	358
C–4[a]	Yellow	Brown	0.60	362
C–5[a]	Yellow	Brown	0.66	362
C–7[a]	Yellow	Brown	0.72	362

[a] Aldehyde may be differentiated from ketone by the loss in light absorption of the aldehyde in alkali.

does not separate ketones and aldehydes of the same chain length. The light absorption characteristics of ketones and aldehydes of the same chain length also are identical. Identification of aldehydes and ketones was found possible by utilizing the method of Huelin *(7)*. In this procedure, the change in light absorption upon aging of the hydrazone in alkali was observed in the spectrophotometer. The aldehyde hydrazones showed a decreased light absorption upon aging, whereas the ketone hydrazones showed no change in absorption characteristics. A combination of color in alkaline solution, R_f values, light absorption characteristics *(1, 11)*, and changes in absorption with aging were utilized for identification.

Application of procedure to cheese. Typical six-month-old commercial Cheddar, Swiss, Camembert, and Blue cheeses were analyzed for acidic and neutral carbonyl compounds by the procedures as outlined above. Compounds present in each cheese were identified on the basis of the preceding methods. Chromatograms of the keto acids for Swiss and Camembert cheese are shown in (Figure 6). The results for acidic and neutral carbonyl compounds in all four cheeses are shown (Table 3). Sixteen different keto acids were found, but only 11 different keto acids were present in any one cheese. Pyruvic and alpha-ketoglutaric acids were major components of all cheeses. Beta-keto acids, which could be derived from fatty acid oxidation, were present only in Blue cheese, whereas alpha-keto acids derived from amino acids were present in highest concentration in Camembert cheese.

TABLE 3

Acidic and neutral carbonyl compounds in four different varieties of cheeses

Carbonyl compounds	Relative concentration of phenylhydrazones in the following cheeses[a]			
	Cheddar	Swiss	Blue	Camembert
Acidic				
1. Oxalsuccinic	−	Trace	Trace	+
2. Alpha-ketoglutaric	2+	3+	2+	2+
3. Oxalacetic	Trace	Trace	Trace	+
4. Gloxylic and isomer			Trace	+
5. Pyruvic and isomer	4+	4+	3+	3+
6. Acetoacetic	−	−	3+	−
7. Alpha-acetolactic	2+	3+	+	Trace
8. Alpha-ketobutyric	−	−	−	−
9. Beta-ketoisocaproic	−	−	2+	−
10. Beta-ketocaprylic	−	−	2+	−
11. Beta-ketocaproic	−	−	2+	−
12. Alpha-ketoisovaleric	+			+
13. Alpha-ketoisocaproic	−	−	−	2+
14. Alpha-hydroxylphenyl pyruvic		−	−	Trace
Neutral				
1. Formaldehyde	−	−	Trace	−
2. Diacetyl	Trace	Trace	Trace	Trace
3. Acetaldehyde	+		+	2+
4. Acetylmethylcarbinol	Trace	Trace	Trace	Trace
5. Acetone	Trace		4+	Trace
6. C–4	++		2+	−
7. Pentanone-2	−		+	−
8. C–5	−		−	+
9. C–7			Trace	

[a] Indicates absence of compound on chromatogram.

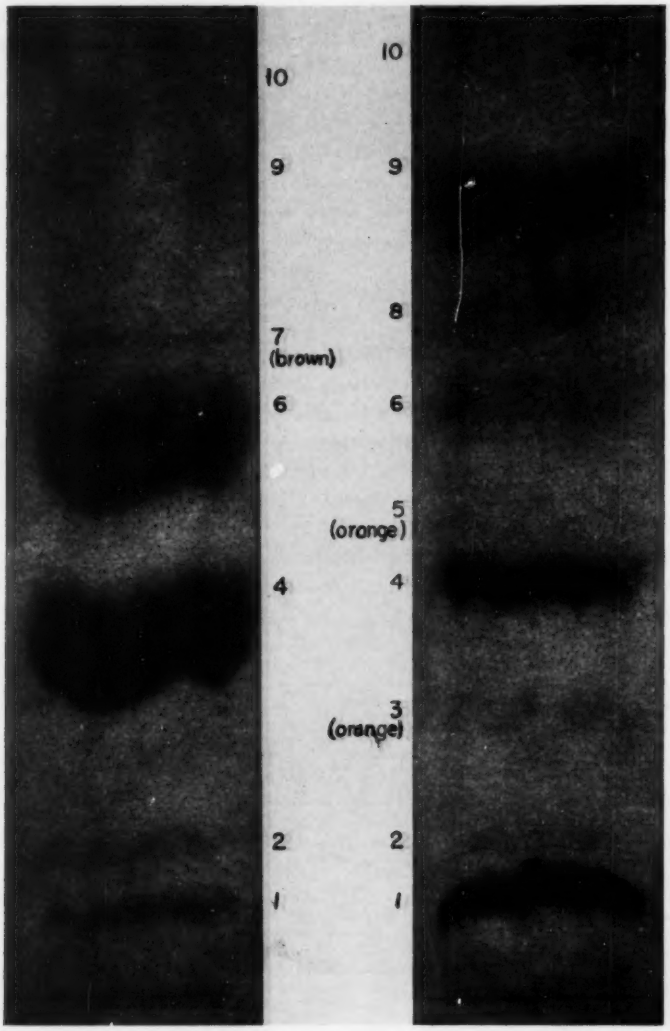

FIG. 5. Chromatograms of keto acid phenylhydrazones in Swiss (left) and Camembert (right) cheeses.

Alpha-acetolactic acid, which is the immediate precursor of acetylmethylcarbinol, was present in all cheese but Camembert. It was identified by its R_f value, brown color in basic solution, and the formation of acetylmethylcarbinol on decarboxylation. The absorption spectra of the alpha-acetolactic acid hydrazones isolated from Cheddar, Swiss, and Blue cheeses are shown (Figure 6), showing maximum absorption at 530 mμ.

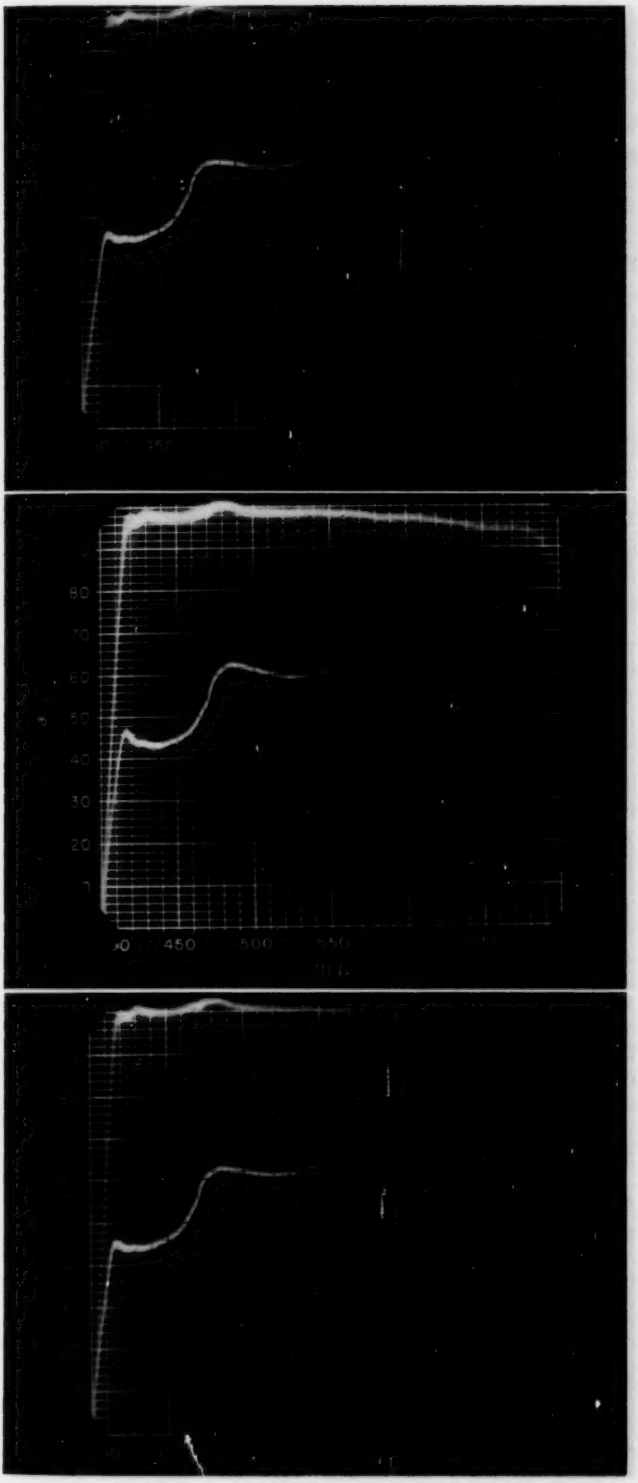

FIG. 6. Visual light absorption patterns of alpha-acetolactic acid phenylhydrazone bonds from Cheddar (top), Swiss (middle), and Blue (bottom) cheeses.

In Swiss cheese, pyruvic acid and alpha-ketoglutaric acid were found as major carbonyl components in all samples analyzed. In addition, oxalsuccinic acid, oxalacetic acid, alpha-acetolactic acid, diacetyl, and acetylmethylcarbinol, were found.

Cheddar cheese contained pyruvic acid, alpha-ketoglutaric acid, oxalacetic acid, alpha-acetolactic acid, alpha-ketoisocaproic acid, diacetyl, and acetylmethylcarbinol.

Blue cheese contained pyruvic acid, alpha-ketoglutaric acid, oxalsuccinic acid, oxalacetic acid, glyoxylic acid, acetoacetic acid, alpha-acetolactic acid, beta-ketoisocaproic acid, beta-ketocaprylic, and beta-ketocaprie acids. The neutral compounds in this cheese were: diacetyl, acetaldehyde, acetylmethyl carbinol, acetone, pentanone-2, and higher unidentified compound(s) following the solvent front.

Camembert cheese contained pyruvic acid, alpha-ketoglutaric acid, oxalacetic acid, glyoxylic acid, alpha-ketoisovalerie acid, alpha-ketoisocaproic acid and phenyl pyruvic acid, acetaldehyde, acetone, and a C-5 aldehyde or ketone.

For each neutral carbonyl compound present in cheeses studied there was a correspondng possible immediate precursor in the group of acidic carbonyl compounds.

Effect of heat on the beta-keto acids of cheese. The absence of heptanone and nonanone in the neutral carbonyl fraction of the Blue cheese suggested that the cheese was atypical or that the method used gave different results from those used previously. These methods have used steam distillation *(4, 10)*, whereas no heat was used in the method reported in this paper. It was found that if the cheese was heated to 100° C. for 10 min., then prepared for chromatographic analyses as outlined, definite measurable quantities of the nonanone and heptanone could be found in cheese with a corresponding decrease in the concentration of beta-keto acids found in that cheese.

Alpha-acetolactic acid was not found after steam distillation, either in residue or distillate. After steam distillation, there was an increase in the concentration of the neutral carbonyl fraction, which was identified as acetylmethylcarbinol.

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OBSERVATIONS ON THE REDUCTION OF 2,3,5–TRIPHENYLTETRAZOLIUM CHLORIDE BY HOMOFERMENTATIVE LACTIC ACID BACTERIA[1]

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SUMMARY

Triphenyltetrazolium chloride (TTC) acted as an inhibitor of lactic acid production in three of four homofermentative lactic acid bacteria cultures studied. The concentration of TTC causing inhibition varied with the different cultures. An increase in pyruvic acid and a decrease in lactic acid occurred when TTC was reduced by three of the four cultures. *Streptococcus liquefaciens* also reduced TTC to a small extent, but was not inhibited by the TTC concentrations used. This indicates the possibility of a metabolic pathway for lactic acid production different from the other three organisms investigated, or a cell relatively impermeable to TTC. TTC reduction was inhibited by concentrations of iodo-acetic acid as low as 10^{-4} molar in some cultures. These results indicate that the DPNH reduces the TTC to the formazan rather than reducing pyruvic acid to lactic acid, as normally occurs in glycolysis by homofermentative lactic cultures.

Tetrazolium salts are useful in bacteriological research. They have been used in demonstrating bacterial dehydrogenases *(10)*, in staining microorganisms *(8)*, in tests for bacterial inhibition by antibiotics *(6)*, and to determine the bacterial quality of milk *(5)*.

The tetrazoles are reduced by actively metabolizing bacterial cells to colored water-insoluble formazans. In contrast to methylene blue and resazurin reduction, tetrazole reduction occurs within the cell *(4)*. The reported oxidation-reduction potential for 2,3,5–triphenyltetrazolium chloride (TTC) is −0.08 V *(9)*. This falls in the proper range for demonstration of many enzymatic oxidation-reduction systems *(10)*. Since hydrogen ions are necessary for the reduction of TTC to formazan, the enzymes involved logically would be the dehydrogenase coenzyme systems and their specific hydrogen acceptors. The conversion of lactose to lactic acid by homofermentative lactic acid bacteria through glycolysis involves one such dehydrogenase coenzyme system which reduces pyruvic to lactic acid.

The oxidation-reduction potentials for TTC and pyruvic acid reduction are similar. Kinetically, it should be possible for TTC to be reduced competitively with pyruvic acid, assuming entry into the cell and, thereby, to depress lactic acid formation. In this instance, an increase in pyruvic acid should occur, since the main pathway for its disposal is partially blocked.

The experiments in this study were designed specifically to investigate the following:

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1. To determine if TTC reduction caused an increase in pyruvic acid concentration and a decrease in lactic acid production in lactic cultures.
2. To determine the concentration of TTC necessary in the medium to inhibit growth of various lactic cultures.
3. To determine if various concentrations of iodo-acetic acid, when incorporated in the medium, would inhibit TTC reduction by lactic cultures.

METHODS

Cultures of *Streptococcus lactis, Streptococcus thermophilus, Streptococcus liquefaciens,* and *Lactobacillus bulgaricus* used in the study were obtained from the Department of Bacteriology, University of Wisconsin. A 10% reconstituted nonfat dry milk medium, sterilized in the autoclave at 121° C. for 15 min., was used for daily transfers and growing cells for acid determinations. *S. lactis* was incubated at 32° C., the other three cultures were incubated at 37° C. TTC[3] solutions, in two concentrations, were made up in sterile distilled water; one contained 1 g. of TTC in 24 ml. of water and the second contained 1 g. of TTC in 99 ml. of water.

Experiments to determine the effect of TTC on bacterial growth and acid production were done in the following manner:

1. One ml. of active culture diluted 1:1 with sterile 10% reconstituted nonfat dry milk was placed in each of a series of sterile test tubes.
2. Two tubes were designated as the controls. Various known amounts of the TTC solution were added to the remaining tubes. Duplicate tubes were made at each concentration of TTC.
3. Sterilized 10% reconstituted nonfat dry milk medium was added to bring all tubes up to a volume of 10 ml.
4. Sterile rubber stoppers were inserted and the tubes inverted twice to mix the contents.
5. The tubes and contents were incubated in a covered water bath at 37° C. Incubation times of 2.5–4.5 hr. were used.
6. After the desired period of incubation, the tubes were removed and immersed in an ice-water bath to stop bacterial growth.
7. The color developed in all tubes was compared to the control tube as a rapid check for increased TTC reduction.
8. On one set of the duplicate tubes, total numbers of viable organisms were determined using a standard plate count procedure *(1)* with tryptone glucose extract agar plus 0.5% yeast extract (Difco) as the plating medium; then the pH was measured with a Beckman Model H-2 pH meter.
9. The other set of duplicate tubes was used to prepare the cell extract as follows:
   a. Five ml. of the growth medium were pipetted into 25 ml. of 10% trichloroacetic acid contained in plastic centrifuge tubes. The contents of the tubes were then mixed well.
   b. After 2 min., the samples were centrifuged at 3,000 r.p.m. in a Model L International Clinical Centrifuge for 10 min. to remove the precipitate.

[3] Obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

c. The clear supernatant was pipetted off and used for lactic and pyruvic acid determinations.

The pyruvic acid was quantitatively determined using essentially the method proposed by Friedman and Haugen *(2)* for determining pyruvic acid levels in blood samples. Six recovery trials with the procedure yielded an average recovery of 99.2%.

Lactic acid in the cell extract was determined by the method of Neish *(7)*. Recovery values obtained for this procedure averaged 99.8%. The developed color for both the lactic and pyruvic acid determinations was measured in a Bausch and Lomb "Spectronic 20" colorimeter.

In the experiments to determine the inhibitory effect of iodoacetic acid (IA) on TTC reduction, the final molar concentrations of IA ranged from 10^{-1} to 10^{-7}. The cells were grown as previously explained, with the IA added to the medium before incubation, any sample having less developed color after incubation than the control indicating inhibition of TTC reduction by the added IA.

RESULTS AND DISCUSSION

The effect of TTC on lactic and pyruvic acid formation is shown (Figures 1 and 2). As the TTC concentration in the medium was increased, an increase

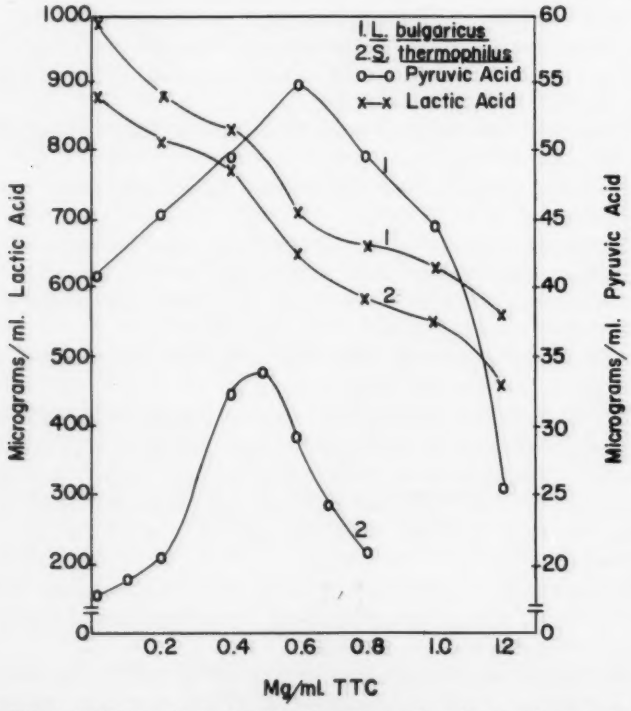

FIG. 1. Effect of TTC on the level of lactic and pyruvic acids in cultures of *S. thermophilus* and *L. bulgaricus* after 4.5 hr. of incubation at 37° C.

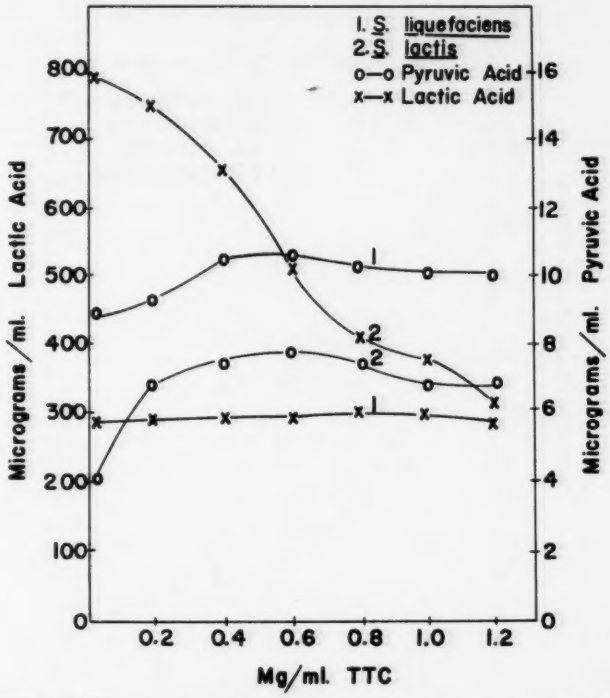

FIG. 2. Effect of TTC concentration on the levels of pyruvic and lactic acids in cultures of *S. lactis* and *S. liquefaciens* after 4.5 hr. of incubation at 37° C.

in pyruvic acid and a decrease in lactic acid occurred in three of the four cultures studied. The *S. thermophilus* and *L. bulgaricus* cultures gave similar reactions; the increase in pyruvic acid concentration and the increase in formazan formation as indicated by changes in color intensity followed an identical pattern over the various TTC concentrations used. However, the peak in pyruvic acid production was at a TTC concentration of 0.4 mg/ml for *S. thermophilus* and at 0.6 to 0.8 mg/ml for *L. bulgaricus*. The sharpest reduction in lactic acid production also occurred at the TTC concentration, where the peak in pyruvic acid production occurred in both cultures.

The other two cultures studied, *S. lactis* and *S. liquefaciens*, reacted differently. The *S. lactis* culture did not exhibit a large increase in pyruvic acid but did show a definite decrease in lactic acid production. Color intensity over the various TTC concentrations was constant. The *S. liquefaciens* produced only a small amount of color from TTC and was not inhibited by the TTC concentrations used.

The number of viable organisms was reduced with increased TTC concentrations for *S. lactis*, *L. bulgaricus*, and *S. thermophilus*. As shown (Figure 3), the *S. liquefaciens* grew as well in all TTC concentrations used as in the control.

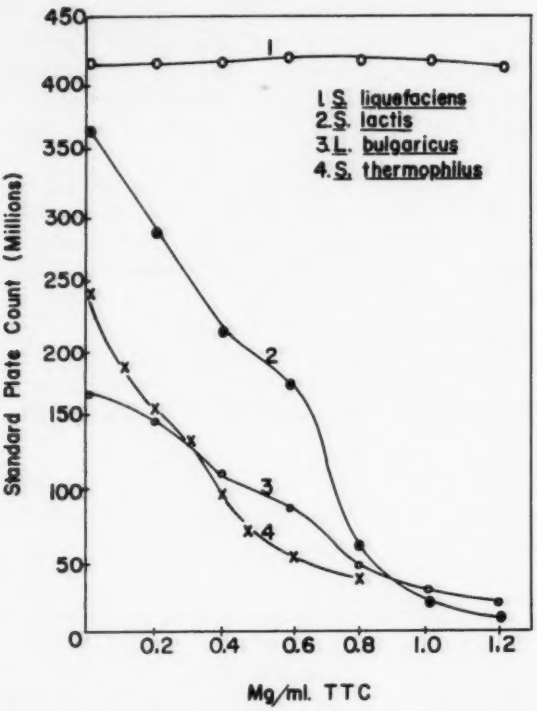

FIG. 3. Effect of TTC concentration on the numbers of viable organisms in various lactic cultures after 4.5 hr. of incubation at 37° C.

This explained the constant level of lactic acid production by this organism in all of the TTC concentrations used. The other three organisms showed both a decrease in lactic acid and in viable numbers at higher TTC concentrations. The presence of higher than normal levels of pyruvic acid, due to some toxic effect, may have contributed some to the reduction in numbers of viable organisms.

This inhibition of growth was probably due to the presence of the water-insoluble formazan within the bacterial cell. Laxminarayana and Iya *(3)* have shown that *S. liquefaciens* is able to destroy triphenyltetrazolium bromide upon extended periods of incubation. This may explain why *S. liquefaciens* was not inhibited by the TTC concentrations used. Another possibility would be that *S. liquefaciens* has more than one pathway for lactic acid production or a pathway different from the other organisms studied.

Iodo-acetic acid (IA) has been used as an inhibitor of reduced diphosphopyridine nucleotide (DNPH) formation in studying the Embden-Myerhoff pathway of carbohydrate metabolism *(11)*. The IA inhibits the action of glyceraldehyde 3-phosphate dehydrogenase and prevents DPN from being reduced.

In trying to confirm that the lactic dehydrogenase–DPN system was involved in TTC reduction, cells were grown with both TTC and various levels of IA in the medium.

The four cultures showed inhibition of TTC reduction with various concentrations of IA. *S. thermophilus* was the most sensitive to IA and a final concentration in the medium of 10^{-6} molar caused inhibition of TTC reduction. An IA concentration of 10^{-5} molar inhibited TTC reduction by *S. liquefaciens*, whereas a concentration of 10^{-4} molar was necessary to prevent TTC reduction by *S. lactis* and *L. bulgaricus*.

ACKNOWLEDGMENT

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CALCIUM EXCRETION IN HUMAN SUBJECTS AT TWO LEVELS OF MILK INTAKE [1]

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SUMMARY

Eight male subjects were maintained for ten days on a low-nitrogen basal diet, supplemented with (1) milk to provide 91% of the total calcium intake, or (2) milk to provide 76% of the calcium intake and eggs to provide 7%. The apparent absorption of calcium when milk was fed at the higher level was 62 ± 8%, and at the lower level, 70 ± 8%. The per cent absorption of total calcium was slightly higher when milk was fed at the lower level; this difference was not significant. Fecal bulk on the dry weight basis increased by 77% when milk was fed at the higher level and fecal calcium increased by 119%; 45–54% of the additional 1.25 g. calcium at the higher level was absorbed.

In metabolic experiments conducted in this laboratory with human subjects, it was observed that when milk was fed in amounts calculated to supply 93% of the body's daily protein intake, there was a concomitant increase in fecal bulk and moisture content of feces. When milk was fed in amounts calculated to supply 45% of the body's protein intake and supplemented with whole egg to supply 48%, fecal bulk and moisture were normal. The purpose of the experiments in progress at the time was to determine the availability of the essential amino acids in milk. However, since milk is a highly recommended dietary source of calcium, it was of interest to determine if the increased fecal bulk was accompained by an increased fecal excretion of calcium.

Sherman *(24)* has reported from studies with man and rats that milk calcium is generally more available than is plant calcium. However, several investigators *(3, 5, 11, 20, 23, 25)* have reported the extravagance of both children and adults with regard to the utilization and retention of milk calcium. According to Lengemann *et al. (13)*, a possible explanation for low values is that the calcium source to be tested is usually incorporated into a well-balanced diet, and the results express availability of dietary calcium, as a whole, rather than of milk calcum. Both Blau *et al.* and Lengemann *et al. (2, 13)* have indicated that the absorption of calcium from foods can not be directly determined by metabolic balance as the difference between dietary calcium and excreted calcium. The feces contain calcium which is secreted with the digestive juices into the gastrointestinal tract (endogenous fecal calcium) in addition to the calcium of the food which passes unabsorbed. Therefore, when the results of conventional balance experiments are used, the true digestibility is underestimated by appreciable amounts. Lengemann *et al. (13)* reported that milk increased calcium absorption in man; from experimentally determined Sr^{85} absorption data, it was estimated that the absorption of milk calcium approached 90–100%. Robinson

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et al. (21) observed that lactose enhanced calcium absorption in calves, and Wasserman *et al. (26)* reported similar observations with rats.

EXPERIMENTAL PROCEDURE

Eight male students in the School of Veterinary Medicine, Tuskegee Institute, were subjects. They were between 23 and 27 years of age and weighed between 61.8 and 77.7 kg. Medical histories revealed no chronic or recent illnesses of the subjects. Neither overt symptoms of nutritional deficiencies nor minor signs attributable to an inadequate intake of a particular nutrient were observed. On the basis of a physical examination and chemical analyses of blood, urine, and feces, each subject was considered to be in good health. There were no changes in the usual activities of these students, except that they came three times daily to the diet kitchen of the School of Home Economics for meals.

A basal diet consisting of an assortment of low-nitrogen fruits and vegetables was fed along with sucrose, butterfat, and milk, or milk supplemented with egg. The basal diet contained 0.90 g. N, 0.30 g. Ca, 116 g. fat, 309 g. carbohydrate, and 2,322 Cal. Milk was fed with or without the egg supplement in amounts calculated to provide a protein intake in the total diet of 1 g/kg body weight. The volumes of milk consumed daily ranged from 1,982–2,130 ml. for subjects who received 93% of their protein in the form of milk, and from 862–1,114 ml. for subjects who received 45% milk protein and 48% egg protein. Calcium intakes in all cases were in excess of the recommended allowance. Sucrose and butterfat were included in amounts to provide a caloric intake in the total diet of 43 Cal/kg/day for R. J. and C. W., 45 Cal/kg/day for L. C., A. C., E. B., and O. C., and 47 Cal/kg/day for D. H. and R. D.

The experimental period was ten days. The first four days were allowed for adjustment to the diet. During the last six days, fecal and urinary excreta were collected. Carmine (Alum Lake) was used as the fecal marker.

Creatinine and total nitrogen determinations were made daily on 24-hr. urine collections. A composite of the six-day fecal collections was lyophilized and assayed for total nitrogen and calcium. The basal diet, milk, and eggs were lyophilized and assayed singly for total nitrogen and calcium. Creatinine was determined according to the procedure of Clark and Thompson *(4)*. The macro-Kjeldahl procedure was used for total nitrogen determinations. Calcium determinations were made on ashed food and fecal samples, according to the procedure of Patton and Reeder *(19)*.

RESULTS AND DISCUSSION

Metabolic data for the eight subjects are presented (Table 1). The average body weights for the two groups of subjects were similar. At the 93% level of milk protein intake, the average fecal bulk on the dry weight basis was 77% higher than at the 45% level. Per cent moisture in feces was also higher at the 93% level. Fecal ash was 121% higher at the higher level of milk intake.

Nitrogen data show that all subjects were in positive nitrogen balance. However, the average nitrogen balance was less positive for the subjects at the 93%

TABLE 1

Metabolic Data

Subject	Body weight	Fecal bulk	Fecal ash	Nitrogen				Calcium						
								Dietary					Fecal Apparent absorption	
				Dietary	Urinary	Fecal	Balance	Milk[a]	Egg[b]	Basal diet	Total	Fecal		
	(lb.)	*(g. dry wt.)*		*(g)*									*(%)*	
Milk, 93% level														
D. H.	164	50.3	10.42	12.61	7.89	1.84	+2.88	2.818	None	0.295	3.113	0.935	2.178	70.0
A. C.	156	51.5	8.04	10.90	8.28	1.74	+0.88	2.623	None	0.295	2.918	1.319	1.599	54.8
E. B.	154	57.1	9.14	11.02	7.72	2.19	+1.11	2.652	None	0.295	2.947	1.148	1.799	61.0
Average	158	53.0	9.20	11.51	7.96	1.92	+1.62	2.698	None	0.295	2.992	1.134	1.859	62±8[c]
Milk, 45% level														
R. J.	176	39.0	4.92	12.55	8.80	1.50	+3.25	1.474	0.129	0.295	1.898	0.605	1.293	68.1
C. W.	175	30.6	4.09	12.38	9.55	1.25	+1.58	1.432	0.126	0.295	1.853	0.560	1.293	69.8
R. D.	156	20.1	3.29	12.08	7.05	0.90	+4.13	1.303	0.114	0.295	1.712	0.420	1.292	75.5
L. C.	158	32.6	4.43	11.95	8.74	1.19	+2.00	1.286	0.113	0.295	1.694	0.700	0.994	58.7
O. C.	138	27.3	4.14	10.69	6.10	1.46	+3.13	1.141	0.100	0.295	1.536	0.300	1.236	80.5
Average	161	29.9	4.17	12.13	8.05	1.26	+2.82	1.327	0.116	0.295	1.739	0.517	1.222	70±8[c]

[a] Calcium content of milk:186.4 mg/g ash.
[b] Calcium content of eggs:45.5 mg/g ash.
[c] Standard deviation.

level of milk intake than for those at the 45% level. Dietary nitrogen per kilogram body weight was the same for both groups. The average fecal nitrogen was 52% higher for men who ingested milk at the 93% level than for those at the 45% level. Urinary nitrogen values for the two groups were quite similar.

Calcium intakes were in all cases in excess of the recommended allowance. For the group at the 93% level of milk intake, 9% of the dietary calcium was contributed by the basal diet and 91% by milk. For the group at the 43% level of milk intake, the basal diet contributed 17% of the calcium, egg 7%, and milk 76%.

The average fecal calcium excreted was 119% higher for the group that received milk at the 93% level of protein intake than for the group at the 45% level. These data agree with findings of several investigators *(2, 12)* who have reported an increase in stool calcium with an increase in calcium intake. They agree also with the 121% increase in fecal ash during the period of higher milk intake. Endogenous fecal calcium was not determined and, therefore, the value of these data is relative. Blau *et al. (2)* report that the endogenous fecal calcium is independent of the level of calcium intake, for short-term experiments. Therefore, endogenous fecal calcium is a bias factor which is constant in both group averages. Urinary calcium determinations were not made. Several investigators *(2, 12, 18)* have reported that urinary calcium remains constant or rises only slightly with marked increases in calcium intake. Urinary level is governed to a much greater extent by endogenous factors than by calcium intake, other factors being identical *(18)*.

Apparent calcium absorption (reported in Table 1) is dietary calcium minus fecal calcium; this difference is not a measure of true absorption but is a composite of ingested calcium plus secreted calcium minus absorbed calcium minus reabsorbed calcium. These values are, therefore, referred to as apparent absorption. The average apparent calcium absorption and the standard deviation for the group at the 93% level was $62 \pm 8\%$ and, for the group at the 45% level, $70 \pm 8\%$. These values indicate that the apparent absorption of total calcium was slightly higher when milk was fed at the lower level, but the difference is not a significant one. However, the apparent absorption of the additional 1.25 g. of milk calcium at the 93% level, rather than the total dietary calcium at this level, is between 46–55%, thus showing a decrease in absorbability at the higher level. Our values for apparent absorption are considerably higher than absorption values reported by Schofield *et al. (22)*; apparent absorption values calculated from their data are 35% for females receiving 1.5 g. calcium, 78% of which was contributed by milk; and 29% for females receiving 1.0 g. calcium, 65% of which came from milk and eggs.

The effects of previous history and present nutritive status on calcium utilization are important in the interpretation of metabolic calcium data. Considerable data are available from animal studies, and some from studies on human subjects, to indicate that excretion of calcium becomes low and retention high after an extended period on a low-calcium diet *(10, 16)*. Macy *(14)* observed that Ceylonese children have a more efficient calcium absorption than children who have

received calcium-rich diets over a period of time. Hegsted *et al. (9)* observed a highly efficient utilization of calcium by inmates of a Peruvian prison. Nicholls and Nimalasuriya *(17)* have shown a low-calcium requirement and light efficiency of adaptation in natives of Ceylon. Several investigators *(6, 7, 15)* have shown the relation of poor calcium utilization to a reduction in quality or quantity of the dietary protein; others *(1, 8)* have shown the same effect by a deficiency of one or more of the essential amino acids.

For 30 days prior to the investigation conducted in our laboratory, the subjects received 1 g. Ca per day in the form of natural foods or a combination of natural foods with a dicalcium phosphate supplement. Their dietary habits prior to this 30-day period are not available. It is likely, however, that they received amounts of the various nutrients daily in excess of the requirements, and that they had good calcium reserves.

Steggerda and Mitchell *(25)* report that there is no basis for assuming differences in the biological value of calcium from various milk products. It is assumed, therefore, that these subjects would show similar absorption values for cottage cheese and other milk products.

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HIGH ROUGHAGE SYSTEM FOR RAISING CALVES BASED ON THE EARLY DEVELOPMENT OF RUMEN FUNCTION. VIII. EFFECT OF RUMEN INOCULATIONS AND CHLORTETRACYCLINE ON PERFORMANCE OF CALVES FED HIGH ROUGHAGE PELLETS

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SUMMARY

In three experiments, 86 Jerseys and 17 Holsteins were used in a study of the effects of rumen inoculations, chlortetracycline and other feed supplements, on growth and digestion of calves fed high roughage pellets. The effect of feeding high roughage pellets of different composition, and of rumen inoculations, on establishment of rumen microorganisms, rumen volatile fatty acids, artificial rumen digestion, and rumen pH are presented in a companion paper.

The feeding of high roughage pellets (basically, two parts one-half inches ground alfalfa hay and one part a simple grain mixture made into pellets one-fourth inches in diameter) resulted in earlier dry feed intake, earlier rumen function, and more rapid growth than was observed in previous experiments, in which loose hay and grain were fed separately in a 2:1 ratio.

Growth and feed intake were enhanced when chlortetracycline was included in the pellets. Little effect was observed when chlortetracycline was fed in the milk to 7 wk., then administered by mixing in 1 lb. of warm water from 7 to 16 wk.

No benefit was observed from the combined effects of adding molasses, distillers solubles, calcium phosphate, and brewers yeast to the basal high roughage pellets in two experiments.

Reducing the total protein of the basal high roughage pellets from 14.3 to 12.3% by replacing all of the soybean meal with corn resulted in retarded growth and lower feed intake in both Jerseys and Holsteins. Replacing approximately one-half the alfalfa hay with good timothy hay in the basal pellets had no appreciable effect on growth or feed consumption, despite somewhat lower digestibility of dry matter and cellulose in the pellets that contained timothy hay.

No significant differences in the performance of rumen-inoculated calves compared to uninoculated calves were measured. There was a tendency, though the differences were not statistically significant, for the uninoculated calves to perform better than those that were inoculated. Rumen (cud) inoculation in relation to the high roughage system for raising calves is discussed.

In previous experiments *(4, 5, 6, 13, 14, 15, 17)* involving high roughage feeding of calves, the physical characteristics of the hay fed were observed to influence the age at which they began to eat appreciable amounts and, consequently, the time when normal rumen function developed. Also, considerable selective eating and waste were encountered when harsh, overdried, or stemmy hay was fed in amounts which permitted maximum consumption.

Preliminary experiments *(12)*, aimed at encouraging earlier dry feed intake through increased uniformity of the physical characteristics of the hay, involved

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chopping or grinding through a ¾-in. hammer mill screen. The ground or chopped hay was mixed with a simple grain mixture in a 2:1 hay-to-grain ratio and fed free choice. The dust problem in these mixtures was overcome by soaking between feedings with an equal weight of water. This produced a soft-textured, palatable feed and resulted in greater dry feed intake, especially during the early weeks, than could be accomplished by feeding loose hay free choice with grain concentrates limited to one-half the amount of hay eaten.

In more recent experiments, the high roughage mixtures (two parts hay and one part grain) were pelleted, ¼-in. diameter, and fed as the only dry feed to 12 or 16 wk. of age.

In this study the performance of calves was measured by growth, feed consumption, and feed utilization using ten different batches of high roughage pellets in three experiments. In presenting these data, an effort has been made to point out the over-all performance of calves fed the pelleted high roughage mixtures, as well as the effects on calf performance of rumen inoculations and certain modifications in the basal pelleted mixture. Modifications included the addition of chlortetracycline, the addition of molasses, sources of extra minerals and B complex vitamins, and variations in the level and source of protein.

Data obtained on establishment of certain indicator rumen microorganisms and various measures of rumen function at different ages in these same calves are also presented in a companion paper *(7)*.

EXPERIMENTAL PROCEDURE AND RESULTS

In three experiments, 86 Jersey and 17 Holstein calves were used. The composition and digestibility of the different batches of high roughage pellets are shown (Table 1). The number of calves allotted to the different pellet groups, the designation of the different rations fed and the various treatments used are shown (Table 2). All pellets consisted of two parts hay (ground through a ½-in. hammeer mill screen) and one part grain concentrates. The pellets were cylindrical and ¼ in. in diameter. Mixing and pelleting were done commercially under supervision, using a continuous line mixer and a Sprout-Waldron pelleting machine.

All calves nursed their dams for approximately three days and then were fed whole milk to 7 wk. of age, the amount being gradually reduced to zero during the 7th wk. The pelleted ration was offered free choice after the third day and, from the end of the milk-feeding period to 16 wk., it constituted the only feed, except that in Experiment 2, Group C_1 (Table 1), the pellets were replaced with loose hay and grain, fed in a 2:1 ratio, at 12 wk. of age. Wheat-straw bedding was consumed in undetermined amounts. Water, salt, and bone meal were available free choice, except that no extra bone meal was offered to the calves in Experiment 2.

One-half of the calves in eight out of the ten Jersey pellet groups (Table 2) were inoculated with fresh, warm cud material from older cattle at 2, 3, 4, 5, and 6 wk. of age. The other half were not inoculated and were fed and handled to keep cross-inoculation from the inoculated calves to a minimum.

TABLE 1

Composition and digestibility of the high-roughage pellets

Experiment No.	1			2			3	
Pellet groups	A	B	C	A_1	C_1	D	D_t[a]	D_e
				Ingredients				
Alfalfa hay *(%)*	66.0	65.5	66.0	66.0	66.0	66.7	33.2	66.7
Timothy hay *(%)*	.....	.....	.....	.....	.....	.....	33.5	.....
Ground shelled corn *(%)*	25.2	25.1	16.4	30.3	22.6	25.5	25.5	33.0
Soybean oil meal *(%)*	8.5	8.5	7.0	3.5	2.0	7.5	7.5	.....
Salt *(%)*	0.3	0.3	0.3	0.2	0.2	0.3	0.3	0.3
Aurofac 2A *(%)*[b]	.....	0.56	.....					
Distillers solubles *(%)*	.....	.....	2.5	.....	2.0			
Molasses *(%)*	.....	.....	5.0	.....	5.0			
Monocalcium phosphate *(%)*	.....	.....	0.3	.....	0.2			
Brewers yeast *(%)*	.....	.....	2.5	.....	2.0			
				Average chemical analysis[c]				
Dry matter *(%)*	87.2	87.5	86.2	87.0	88.1	85.7	84.8	85.7
Crude protein *(%)*	16.9	15.8	16.7	14.9	14.6	14.3	13.4	12.3
Cellulose *(%)*	16.1	16.4	16.3	19.1	17.9	18.1	19.0	17.7
Ash *(%)*	8.9	7.5	8.9	7.5	8.0	6.8	6.8	6.4
				Average digestibility				
Dry matter *(%)*	65.6 (4)[e]	65.0 (4)	.....	62.1 (6)	61.6 (6)	64.9 (3)	59.7 (5)	66.5 (3)
Cellulose *(%)*	50.1 (4)	49.8 (4)	.....	34.8 (6)	38.8 (6)	52.4 (3)	47.2 (5)	50.3 (3)
Protein *(%)*	70.0 (4)	67.6 (4)	.....	67.5 (6)	64.2 (6)	59.3 (3)	62.5 (5)	59.5 (3)
TDN *(%)*[d]	59.6	61.0	.....	54.5	57.4	56.0	51.8	58.4
Nutritive ratio[d]	1 : 3.93	.....	.....	1 : 5.40	1 : 5.10	1 : 5.74	1 : 5.09	1 : 6.96

[a] Difficulty encountered in mixing this batch resulted in less grain and timothy hay in the final pellets than was anticipated.

[b] 20 mg. of chlortetracycline/lb pellets.

[c] Samples for analysis were obtained as fed. Results are reported on the air-dry basis.

[d] Calculated from digestion percentages determined from digestion trials using inoculated male calves.

[e] Numbers in parentheses indicate number of trials using male Jerseys 13 wk. of age in pellet Groups A, A_1, and C_1, and 17 wk. of age in pellet Groups D, D_t, and D_e. All calves in both A and B pellet groups were being fed A pellets at the time of the digestion trials (see Table 2).

TABLE 2

Allotment of calves to pellet groups and other treatments in all three experiments

	Experiment 1			Experiment 2				Experiment 3					
Pellet group	*A*	*B*	*C*	A_1	C_1	C_2[a]	C_3[a]	D		D_t		D_c	
Breed of calves	Jersey	Jersey	Jersey	Jersey	Jersey	Jersey	Jersey	Jersey	Holstein	Jersey	Holstein	Jersey	Holstein
Total number of calves	10	10	10	6	10	5	5	10	5	10	7	10	5
No. inoculated	5	5	5	3	5	5	5	5	5	5	7	5	5
No. uninoculated	5	5	5	3	5	—	—	5	—	5	—	5	—
Kind of pellets fed:													
1–8 wk.	A	B	C	A_1[b]	C_1	C_1	C_1	D	D	D_t	D_t	D_c	D_c
8–12 wk.	A	B	A	A_1	C_1	C_1	C_1	D	D	D_t	D_t	D_c	D_c
12–16 wk.	A	A	A	—[c]	—[c]	C_1	C_1	D	D	D_t	D_t	D_c	D_c
Chlortetracycline fed	None	1–12 wk.[d]	None	1–3 wk.[e]	1–3 wk.[e]	None	1–16 wk.[f]	1–7 wk.[d]		1–7 wk.[d]		1–7 wk.[d]	

[a] One-half the calves in all groups except C_2 and C_3 were not inoculated; calves in the C_2 and C_3 groups were all inoculated.
[b] Data for A_1 calves not included after 8 wk. because of disease (see procedure, Experiment 2, Table 3).
[c] Fed loose alfalfa hay and grain in a 2:1 ratio from 12–16 wk.
[d] Chlortetracycline included in pelleted ration (20 mg/lb in form of Aurofac 2A) to 12 wk.
[e] Chlortetracycline fed in milk, 100 mg/day, in form of Aurofac D.
[f] Chlortetracycline fed in milk to 7 wk. and in 1 lb. of warm water 8–16 wk., inclusive (100 mg/day), in form of Aurofac D.

Records were kept of daily feed consumption, weekly changes in body weight, and withers height at 1, 8, 12, and 16 wk. of age.

In all three experiments, five-day digestion trials were conducted using the male Jerseys 13 to 17 wk. of age. During these digestion trials pellets were fed in constant amounts based on consumption immediately prior to the trial, and urine and feces were collected separately, using metabolism crates.

Based on the average pellet consumption of the different pellet groups, and using the digestion percentages experimentally determined, the intake of total digestible nutrients (TDN), TDN per pound of grain, protein digested, digestible protein per pound gain and the nutritive ratos were calculated. The following is a description of the procedure and results of each of the three experiments:

Experiment 1. This experiment was conducted to study the effects of adding to the basal pelleted ration (A pellets) fed to cud-inoculated vs. uninoculated calves either chlortetracycline (B pellets) or extra minerals, B complex vitamins, and molasses (C pellets). Information on the percentage of each ingredient in the pelleted rations and their chemical composition and digestibility are shown (Table 1).

Ten calves, born in February, March, and April, 1954. were assigned to each pellet group, one-half of which were inoculated (Table 2). Limited whole milk was fed, using open buckets, to 7 wk. of age at the rate of 1 lb. per 10 lb. birth weight daily. High roughage pellets were offered free choice to all calves beginning on the fourth day of age. In the A group, basal A pellets were fed free choice to 16 wk. of age.

The calves in the B group were fed B pellets containing 20 mg. of aureomycin (Aurofac 2A) per pound of dry feed to 12 wk. of age. From 12–16 wk. these calves were fed A pellets (Table 2).

The C group was fed C pellets to 8 wk. of age, after which they were fed the basal A pellets to 16 wk. (Table 2). The purpose here was to study the possible combined effects of the added molasses, distillers solubles, calcium phosphate, and brewers yeast (see Table 1) on early rumen development *(7)* and calf performance.

As only small, statistically insignificant differences existed in average performance of cud-inoculated vs. uninoculated calves, the data for both were combined and are shown Table 3 and Figure 1). It will be noted that the average gain of the three groups to 16 wk. of age was relatively high, 115 lb. compared to 90 lb. for the Ragsdale *(22)* standard and 107 lb. for the Cornell *(24)* standard.

Because of a disease problem which resulted in chronic pneumonia and poor performance in the cud-inoculated calves in the C pellet group (averge gain to 16 wk. only 73 lb. compared to 120 lb. for the uninoculated calves), performance data for the five inoculated calves are not included in the averages shown (Table 3). It will be noted (Table 3) that the uninoculated calves fed the C pellets to 8 wk. compared favorably in performance with the calves in the C_1 and C_2 pellet groups in the next experiment, where a similar ration was fed (Table 1).

TABLE 3

Average performance of Jersey calves fed high roughage pellets to 16 wk. of age

	Experiment 1			Experiment 2					
Pellet group[a]	A	B	C	A_1	C_1	C_2	C_3		
No. of calves	10	10	5	6	10	5	5		
Initial wt. 1 wk. *(lb.)*	51	50	53	54	61	53	51		
Wt. gain 8 wk. *(lb.)*	35	43	41	45	42	39	45		
Wt. gain 8–12 wk. *(lb.)*	29	41	35	20[c]	32	37	37		
Wt. gain 12–16 wk. *(lb.)*	41	36	44	.....	35	37	37		
Wt. gain 8–16 wk. *(lb.)*	70 ±17.6*	77 ±11.8	79 ±23.5	.....	67 ±12.6	74 ± 6.1	74 ± 9.5		
Wt. gain 16 wk. *(lb.)*	105	120	120	....	109	113	119		
Initial withers ht. 1 wk. *(in.)*	25.7	25.9	26.4	25.3	26.0	24.9	25.2		
Withers ht. gain 8 wk. *(in.)*	2.7	2.6	2.1	2.3	2.8	2.9	4.0		
Withers ht. gain 8–12 wk. *(in.)*	1.3	2.2	1.7	2.2[c]	1.3	1.2	1.1		
Withers ht. gain 12–16 wk. *(in.)*	2.1	1.9	2.2	.....	2.2	1.9	1.4		
Withers ht. gain 8–16 wk. *(in.)*	3.4 ± 0.67	4.1 ± 0.33	3.9 ± 0.78	.....	3.5 ± 0.92	3.1 ± 1.16	2.5 ± 0.84		
Withers ht. gain 16 wk. *(in.)*	6.1	6.7	6.0	.....	6.3	6.0	6.5		
Whole milk 7 wk. *(lb.)*	215.5	220.8	221.1	296.5[h]	295.9[h]	287.0	284.0[h]		
High roughage pellets 8 wk. *(lb.)*	42.5	58.5	57.2	56.4	67.5	55.0[g]	72.9[g]		
High roughage pellets 8–12 wk. *(lb.)*	103.4	135.7	126.7[e]	102.5[c]	128.8	120.5[g]	127.8[g]		
High roughage yellets 12–16 wk. *(lb.)*	151.4	160.7[d]	186.9[e]	..... [f]	198.7[f]	180.0[g]	160.7[g]		
High roughage pellets 8–16 wk. *(lb.)*	254.3 ±53.5	296.4 ±48.4[d]	313.6 ±80.5[e]	..... [f]	327.5 ±52.5[f]	300.5 ±13.6[g]	288.7 ±51.2[g]		
High roughage pellets 16 wk. *(lb.)*	297.3	354.9	370.8	..... [f]	394.5[f]	355.5[g]	361.6[g]		
TDN intake 8 wk. *(lb.)*	59.0	62.8	69.9	79.0	87.4	78.4	88.1		
TDN intake 8–12 wk. *(lb.)*	61.6	82.8	75.5	55.9[c]	73.5	69.2	73.4		
TDN intake 12–16 wk. *(lb.)*	86.3	97.8	111.3	.....	114.2[f]	103.3	192.3		
TDN intake 8–16 wk. *(lb.)*	147.9 ±30.2	180.6 ±29.5	186.8 ±51.8	.....	187.7 ±30.2[f]	172.5 ±11.3	165.7 ±29.5		
TDN intake 16 wk. *(lb.)*	206.9	243.4	256.7	.....	275.1[f]	250.9	253.8		
TDN/lb gain 8–16 wk. *(lb.)*	2.14	2.35	2.37	..... [e]	2.80	2.33	2.24		
TDN/lb gain 16 wk. *(lb.)*	1.97	2.03	2.14	.....	2.52	2.22	2.13		
Pellets/lb gain 8–16 wk. *(lb.)*	3.64	3.85	3.97	.....	4.89	4.06	3.90		
Protein digested 8–16 wk. *(lb.)*[b]	30.0	33.5[d]	37.1[e]	..... [e]	.....	28.2	27.1		
Digested protein/lb gain 8–16 wk. *(lb.)*[b]	0.43	0.44[d]	0.47[e]	.....	.....	0.38	0.37		

* Standards deviations.

[a] One-half the calves in each pellet group except C_2 and C_3 were not inoculated. All calves in the C_2 and C_3 groups were inoculated. Data for the uninoculated calves *only* are reported in the C pellet group (see procedure, Experiment 1).

[b] Av. based on actual digestibility data (Table 1).

[c] Data for A_1 calves not included after 12 wk. Four calves were removed from experiment between 12 and 16 wk. because of disease (see discussion).

[d] B pellets replaced with A pellets at 12 wk.; thus, no chlortetracycline was fed after 12 wk.

[e] A pellets replaced C pellets at 8 wk.

[f] A_1 and C_1 calves were fed loose hay and grain at a 2:1 ratio from 12–16 wk.; thus, these figures include the loose hay and grain fed.

[g] Both C_2 and C_3 groups were fed C_1 pellets throughout the experiment.

[h] 100 mg. chlortetracycline (Aurofac D) per day fed in the milk to 3 wk. of age in Groups A_1 and C_1 and to 7 wk. in Group C_3. Chlortetracycline feeding was continued in Group C_3, 8–16 wk., 100 mg. in 1 lb. of warm water once daily.

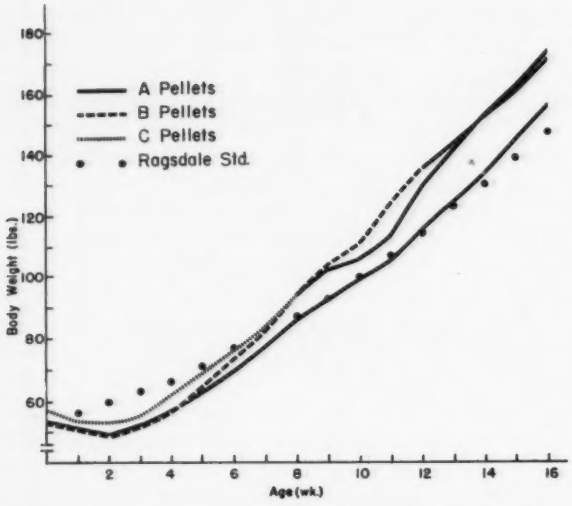

FIG. 1. Average weekly body weight changes of calves in Experiment 1. In the B group, B pellets were fed to 12 wk. followed by A pellets from 12–16 wk. In the C. group, C pellets were fed to 8 wk., followed by A pellets from 8–16 wk. Only the five uninoculated calves were included in the C group.

Experiment 2. In Experiment 2, the study was continued of the effects of adding supplemental sugar, minerals, and B complex vitamins in the form of molasses, distillers solubles, monocalcium phosphate and dried brewers yeast to the basal ration.

In contrast to Experiment 1, extra milk was fed to the calves in this experiment during the first 2 wk. The milk-feeding schedule was as follows: First three days, nursed the dam; four to seven days, 5 lb. twice daily; second week. 4 lb. twice daily; 3–6 wk. inclusive, 3 lb. twice daily; 7th wk., gradually reduced to zero. Open buckets were used for milk feeding.

The basal A_1 ration was fed to the A_1 group of six Jersey calves born during October and November, 1954. The A_1 pellets were similar to the A pellets fed in Experiment 1, except that a different batch of alfalfa hay was used and minor changes were made in the grain mixture (Table 1). The calves fed the A_1 pellets were compared with the ten Jersey calves in the C_1 group, which were fed the supplemented C_1 pellets (Table 1). These calves were born during February and March, 1955. As indicated (Table 2) both the A_1 and C_1 groups were fed high roughage pellets to 12 wk. of age, at which time the A_1 group was discontinued and the C_1 group was fed loose hay and a simple grain mixture from 12 to 16 wk. of age. One-half the calves in both the A_1 and C_1 groups were cud-inoculated. As in Experiment 1, both inoculated and uninoculated calves were combined in the performance data (Table 3 and Figure 2).

Both inoculated and uninoculated calves in the A_1 group grew well and at

about the same rate, 45 lb. gain during the first 8 wk. After 8 wk., neither group gained normally because of respiratory infections, the inoculated calves being adversely affected to a greater extent than the uninoculated calves. Up to 8 wk. of age the calves fed the C_1 pellets did not gain quite as well as those in the A_1 pellet group.

After 8 wk., the growth and feed intake data of the calves in the A_1 group were more closely related to their poor health than to the ration fed. Thus, comparisons with the C_1 pellet groups could not be made after 8–9 wk. However, performance data were included for the A_1 group to 12 wk. (Table 3 and Figure 2).

To obtain further information on the effects of feeding chlortetracycline, ten different Jersey calves also born durng February and March, 1954, were divided into two groups of five (C_2 and C_3). All these calves were cud-inoculated (Table 2). The C_3 pellet group was fed 100 mg. of chlortetracycline per day in the form of Aurofac D mixed in the milk to 7 wk. of age. After the milk-feeding period, chlortetracycline feeding was continued to 16 wk. at the same rate by mixing it with 1 lb. of warm water. Open buckets were used to feed milk and also the chlortetracycline, water mixture. This was in contrast to the B pellet group (Experiment 1) in which chlortetracycline was added to the dry feed. The other five calves, C_2 pellet group, served as controls. C_1 pellets were fed to both C_2 and C_3 calves to 16 wk. Thus, the five inoculated calves in the C_2 group were fed the same as the ten calves in the C_1 group, half of which were inoculated. The performance data for the C_2 and C_3 pellet groups are shown

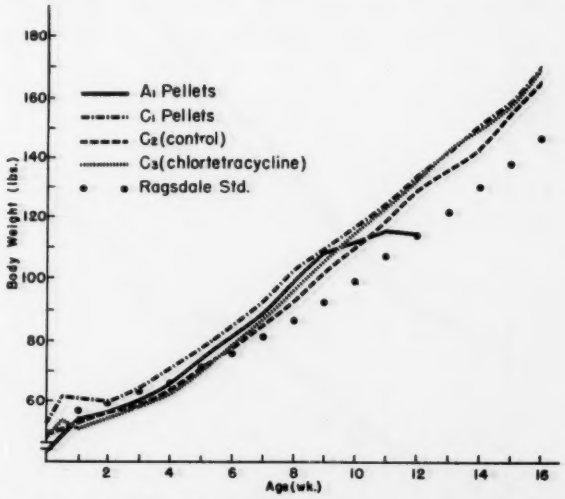

FIG. 2. Average weekly body weight changes of calves in Experiment 2. The calves in the C_1 group were fed loose hay and grain after 12 wk. A respiratory infection in the A_1 group resulted in poor growth after 9 wk. and the calves were removed from experiment at 12 wk. The calves in both the C_2 and C_3 groups were fed C_1 pellets throughout the experiment.

also (Table 3 and Figure 2). As in Experiment 1, the average gain to 16 wk. was 115 lb.

Experiment 3. Experiment 3 was designed to get information on: *(1)* The effect of reducing the total protein in the basal D pellets from 14.3 to 12.3%, either by replacing the soybean oil meal with corn (D_c pellets) or by replacing one-half the alfalfa hay with timothy hay (D_t pellets); *(2)* whether the protein requirement of calves could be satisfied from the hay protein without using a protein supplement, i.e., if the lowered protein did not reduce growth (D and D_t vs. D_c pellets); and *(3)* whether good mixed hay would be as satisfactory as alfalfa hay for use in the pelleted rations (D_t vs. D and D_c pellets). Mechanical difficulties in mixing resulted in the inclusion of less timothy and grain mixture than was anticipated in the D_t pellets. This is reflected in the chemical analysis of the pellets, which were sampled as fed (Table 1).

The calves used in this experiment were all born from August, 1955, to March, 1956. Ten Jersey calves were included in each of the three pellet groups. Also,

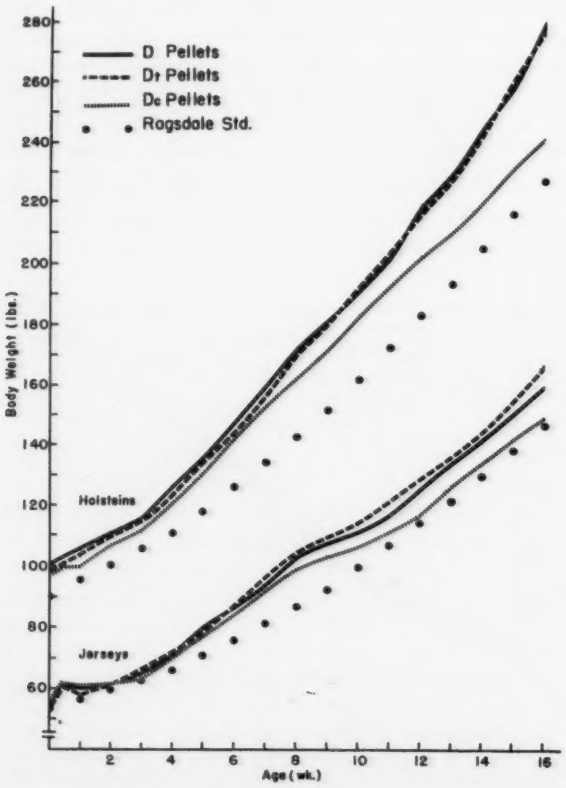

FIG. 3. Average weekly body changes of calves in Experiment 3.

TABLE 4

Average performance of Jersey and Holstein calves fed high roughage pellets to 16 wk. of age

	Experiment 3					
	Jerseys[c]			Holsteins[c]		
Pellet group	D	D_t	D_c	D	D_t	D_c
No. of calves	10	10	10	5	7	5
Initial wt. 1 wk. *(lb.)*	60	58	61	105	103	100
Wt. gain 8 wk. *(lb.)*	43	46	38	67	67	62
Wt. gain 8–16 wk. *(lb.)*	56 ±14.1	62 ±10.3	50 ± 7.1	107 ±18.2	107 ±18.2	79 ±15.0
Wt. gain 16 wk. *(lb.)*	99	108	88	174	174	141
Initial withers ht. 1 wk. *(in.)*	25.6	25.6	26.1	29.1	29.1	29.2
Withers ht. gain 8 wk. *(in.)*	3.3	3.2	2.5	2.8	3.5	3.1
Withers ht. gain 8–16 wk. *(in.)*	3.5 ± 1.16	3.8 ± 0.71	3.3 ± 0.59	4.2 ± 1.24	3.5 ± 1.09	3.3 ± 1.59
Withers ht. gain 16 wk. *(in.)*	6.8	7.0	5.8	7.0	7.0	6.4
Whole milk, 7 wk. *(lb.)*[a]	291.0	292.3	286.4	461	474	462
High roughage pellets 8 wk. *(lb.)*	67.8	66.7	63.0	73	74	81
High roughage pellets 8–16 wk. *(lb.)*	267.5 ±43.1	285.3 ±45.0	228.5 ±20.8	395 ±65.1	398 ±82.6	341 ±28.9
High roughage pellets 16 wk. *(lb.)*	335.3	352.0	291.5	468	472	422
TDN intake 8 wk. *(lb.)*	85.1	80.7	82.8	116	116	123
TDN intake 8–16 wk. *(lb.)*	150.0 ±21.1	147.4 ±23.3	132.6 ±12.1	221 ±39.5	206 ±28.4	199 ±17.3
TDN intake 16 wk. *(lb.)*	235.1	228.1	215.4	337	322	322
TDN/lb gain 8–16 wk. *(lb.)*	2.68	2.38	2.65	2.07	1.93	2.53
TDN/lb gain 16 wk. *(lb.)*	2.38	2.11	2.45	1.94	1.85	2.28
Pellets/lb gain 8–16 wk. *(lb.)*	4.78	4.60	4.57	3.69	3.72	4.32
Protein digested 8–16 wk. *(lb.)*[b]	21.3	24.3	16.8			
Digested protein/lb gain 8–16 wk. *(lb.)*[b]	0.38	0.39	0.34			

[a] 100 mg. Aureomycin (Aurofac D) fed daily in the milk to 7 wk.

[b] Average based on digestibility data (Table 2).

[c] One-half the Jerseys and all the Holsteins were rumen-inoculated.

FIG. 4. Average weekly body weight changes of 30 inoculated vs. 30 uninoculated calves in Experiment 1, 2, and 3 (pellet groups combined were A, B, C_1, D, D_t, and D_c).

17 Holsteins were divided as follows among the three rations: D—five, D_t—seven, and D_c—five. All the Holsteins and one-half of the Jerseys were rumen-inoculated.

In this experiment, the Jersey calves were fed on the same milk-feeding schedule described in Experiment 2, extra milk being fed during the first 2 wk., compared to the more limited milk feeding in Experiment 1. The Holstein calves in this experiment were also fed on an extra-milk schedule during the first 2 wk., as follows: First three days, nursed dam; four to seven days, 8 lb. twice daily; second week, 6 lb. twice daily; 3–6 wk. inclusive, 5 lb. twice daily; 7th wk. gradually reduced to zero. As in the other experiments, open buckets were used for feeding milk.

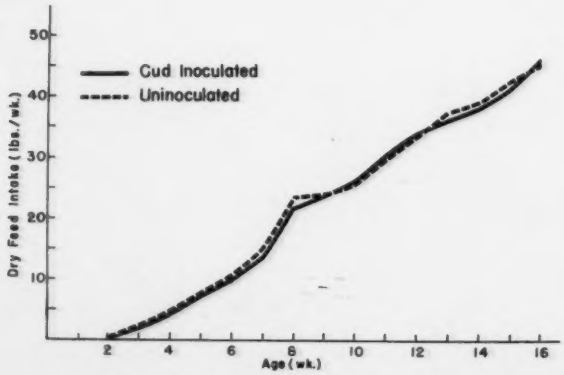

FIG. 5. Average dry feed intake (lb/wk) of 30 inoculated vs. 30 uninoculated calves in Experiments 1, 2, and 3 (pellet group combined were A, B, C_1, D, D_t, and D_c).

All calves in this experiment were fed 100 mg. of chlortetracycline (Aurofac D) per day mixed in the milk to 7 wk. of age. Performance data for both Jerseys and Holsteins are shown separately (Table 4 and Figure 3).

As in the previous experiments, preliminary examination of the Jersey data showed that only relatively small differences in average performance existed between the inoculated and the uninoculated calves in the various pellet groups. Therefore, in Table 4, and in Figure 3, the average performance data of the five inoculated and five uninoculated Jersey calves in each of the three pellet groups were combined.

Rumen-Inoculated vs. uninoculated Jersey calves, Experiments 1, 2, and 3. In order to show the effects of rumen inoculations on the performance of calves fed the pelleted rations, the combined performance data of 30 inoculated and 30 uninoculated Jersey calves are presented (Table 5 and Figures 4 and 5). These data include the five inoculated and five uninoculated calves from the following pellet groups: A and B, Experiment 1; C_1 Experiment 2; D, D_t, and D_c, Experiment 3. Table 6 shows the growth data, both body weight and withers height, for each of the six groups separately.

DISCUSSION OF RESULTS

Results of these three experiments will be discussed first on the basis of the over-all effects of high roughage pelleted rations, on growth and performance,

TABLE 5

Average performance of Jersey rumen-inoculated vs. uninoculated calves[a]

	Rumen-inoculated	Uninoculated
No. of calves	30	30
Initial wt. 1 wk. *(lb.)*	56	58
Wt. gain 8 wk. *(lb.)*	40	43
Wt. gain 8–16 wk. *(lb.)*	61 ±13.1	65 ±18.1
Wt. gain 16 wk. *(lb.)*	101	108
Initial withers ht. 1 wk. *(in.)*	25.9	25.8
Withers ht. gain 8 wk. *(in.)*	2.8	2.9
Withers ht. gain 8–16 wk. *(in.)*	3.6 ± 0.96	3.7 ± 0.84
Withers ht. gain 16 wk. *(in)*	6.4	6.6
Whole milk 7 wk. *(lb.)*	264.5	269.4
High roughage pellets 8 wk. *(lb.)*	54.9	67.3
High roughage pellets 8–16 wk. *(lb.)*	279.6 ±44.5	273.6 ±62.0
High roughage pellets 16 wk. (lb.)	334.5	340.9
TDN intake 8 wk. *(lb.)*	76.5	76.0
TDN intake 8–16 wk. *(lb.)*	157.9 ±25.4	157.5 ±37.5
TDN intake 16 wk. *(lb.)*	234.4	233.5
TDN/lb gain 8–16 wk. *(lb.)*	2.59	2.39
TDN/lb gain 16 wk. *(lb.)*	2.32	2.14
Pellets/lb gain 8–16 wk. *(lb.)*	4.58	4.15
Protein digested 8–16 wk. *(lb.)*[b]	25.4	26.1
Dig. protein/lb gain 8–16 wk. *(lb.)*[b]	.42	.40
Nutritive ratio 8–16 wk.[b]	1: 5.32	1: 5.15
Dry matter digested *(%)*	65.0 (9)[c]	62.0 (10)
Cellulose digested *(%)*	50.5 (9)	49.3 (10)
Protein digested *(%)*	65.2 (9)	63.1 (10)

[a] Five rumen-inoculated calves and five uninoculated calves from each of the following groups were included: A, B, C_1, D, D_t, and D_c.

[b] Averages based on actual digestibility data.

[c] Numbers in parentheses indicate number of digestion trials, using male Jerseys 13–17 wk. of age.

TABLE 6

Average body weight and withers height of rumen-inoculated and uninoculated Jersey calves in the different pellet groups

Pellet group	A	B	C_t	D	D_t	D_c	Average
				Rumen-inoculated			
No. of calves	5	5	5	5	5	5	30
Initial wt. 1 wk. *(lb.)*	49	50	60	60	58	59	56
Wt. gain 8 wk. *(lb.)*	29	45	40	37	47	39	40
Wt. gain 8–16 wk. *(lb.)*	67	71	67	57	59	50	61
Initial withers ht. *(in.)*	25.6	26.0	26.2	25.4	25.7	26.4	25.9
Withers ht. gain 8 wk. *(in.)*	2.5	2.5	2.7	3.0	3.5	2.8	2.8
Withers ht. gain 8–16 wk. *(in.)*	3.2	4.1	3.1	4.0	3.8	3.0	3.6
				Uninoculated			
No. of calves	5	5	5	5	5	5	30
Initial wt. 1 wk. *(lb.)*	52	51	61	61	58	62	58
Wt. gain 8 wk. *(lb.)*	41	41	45	48	46	38	43
Wt. gain 8–16 wk. *(lb.)*	74	84	66	55	63	49	65
Initial withers ht. *(in.)*	25.9	25.8	25.9	25.8	25.4	26.1	25.8
Withers ht. gain 8 wk. *(in.)*	2.8	2.8	2.9	3.6	2.8	2.2	2.9
Withers ht. gain 8–16 wk. *(in.)*	3.6	4.1	3.8	3.0	3.8	3.6	3.7

followed by the effects of chlortetracycline, basal vs. supplemented grain concentrate mixtures, level and source of protein, and rumen inoculations.

The over-all gains of the calves fed high roughage pellets were considerably higher than had previously been obtained when loose hay and grain were fed. Average gain to 16 wk., including the low protein groups in Experiment 3, was 108 lb. compared to 90 lb. for the Ragsdale standard *(22)* and 107 lb. for the Cornell standard *(24)*. While no direct comparisons were made with similar unpelleted rations, it is apparent (Tables 2 and 4 and Figures 1, 2, and 3) that growth exceeded the Ragsdale standard *(22)*. The Ragsdale standard in general was about the average growth rate obtained when rations consisting of loose hay and grain were fed *(6, 13, 14, 17)*, when grain feeding was limited by the amount of hay being consumed.

The favorable effect of the extra-milk feeding during the first 2 wk. in Experiments 2 and 3 compared to Experiment 1 can be seen in the 8 wk. performance data (Tables 3 and 4) and more clearly in the growth curves (Figures 1, 2, and 3). Average increase in total milk fed the Jerseys in Experiments 2 and 3 compared to Experiment 1 was 72.1 lb. The improved performance of the calves in Experiments 2 and 3 during the early weeks indicates that more attention should be gixen to meeting the high energy needs of the young calf, especially during the first ten days. Roy *et al. (23)* have demonstrated that the basal metabolism of the calf is especially high during this period.

Comparison of the feed intake on the pelleted rations (Tables 3 and 4) with that obtained in previous work, in which loose hay and grain were fed separately *(17)*, shows that the calves ate larger amounts of dry feed in pelleted form, especially during the early weeks. Total dry feed intake to 4 wk. of age in calves fed long hay was 4.5 lb. compared to 6.7 lb. when pelleted rations were fed. The over-all increase in feed intake and faster growth of calves fed high roughage pellets is in general agreement with results obtained by others *(10, 18)*, when

pelleted hay was fed to calves and lambs. Other advantages observed in pellet feeding were prevention of selective eating and waste, elimination of dust, and reduced time and labor in feeding.

The digestibility data shown (Table 1) indicate that per cent dry matter digestion and per cent protein digestion of the pelleted rations were in the range previously observed when long alfalfa hay and grain were fed separately *(4, 6, 14)*. However, the per cent cellulose digestion (average 50.1%, Table 1) was lower than the values obtained when long hay was fed, average over 65% *(4, 6, 14)*. This was attributed largely to the fineness of grind which, according to Balch *(1)*, determines in part the rate of passage through the rumen and, consequently, the amount of time available for bacterial cellulose digestion. Based on the results of Blaxter *et al. (2, 3)*, using pelleted grass–fed wethers, the loss in cellulose digested may have been at least partially offset by less methane loss in the pelleted rations. Thus, the increased rate of passage with the accompanying increased feed intake likely accounted for the faster growth of the calves fed the pelleted rations. High roughage pellets made from somewhat coarser roughage than was used in these experiments would have resulted in higher cellulose digestibility *(12)* and might have resulted in even better calf performance.

Effects of feeding chlortetracycline, Experiments 1 and 2. As previously observed *(14)*, the feeding of chlortetracycline did not markedly affect the digestibility of the feed (Table 1, comparing Groups A and B, Experiment 1). Table 3 shows that the calves in pellet Group B, where chlortetracycline was included in the pelleted ration to 12 wk., grew faster during the first 8 wk. ($P < .05$) and also during the 8–12 wk. period ($P < .05$), as measured by both body weight and withers height. After these calves were changed at 12 wk. of age to A pellets, containing no chlortetracycline, growth was slower than the A control group during the 12–16 wk. period (Table 3), although the B calves ate more feed during this period. This resulted in a decreased effiiciency of feed utilization following chlortetracycline feeding, as was also previously observed *(14)*, and is in agreement with the results of Marshall *et al. (19)*.

In Experiment 2, Group C_3, where chlortetracycline was fed mixed in the milk to 7 wk. and in 1 lb. of warm water from 7–16 wk., neither growth nor feed intake was increased except during the first 8 wk. (Table 3), compared with the control calves in Group C_2. This suggests that the method of administration was a factor in the response to chlortetracycline from 8–16 wk., when rumen function was well developed, as indicated by different measures of rumen function *(7)*.

Basal vs. supplemented concentrate mixtures, Experiments 1 and 2. In comparing the performance of calves fed the basal A and A_1 pellets with those fed the supplemented C and C_1 pellets, it is evident that no important over-all advantages resulted from the use of the supplemented grain mixture in the pellets, either in the data shown in Table 3 and Figures 1 and 2, or in the data discussed in the accompanying paper *(7)*. On this basis, it is doubtful that the addition of supplemental molasses, trace minerals and B complex vitamins to

high roughage pellets would result in any measurable benefit. This is in agreement with previously reported results, using calves fed a high roughage diet *(17)*.

Effects of level and sources of protein, Experiment 3. The performance data of the calves in Experiment 3 (Table 4 and Figure 3) show that the D_c ration, when the protein content was lower and nearly all the protein was derived from the roughage, was inferior to both the D and D_t rations, each of which contained some protein supplement and were also higher in protein. It was not possible to determine whether lower total protein content, or the fact that most of the protein was derived from the roughage, was responsible for the decreased growth of the D_c group. However, based on protein requirement in relation to the amount of digestible protein consumed (Table 4), it is apparent that low protein intake alone could account for reduced growth. When both Jerseys and Holsteins were combined the lower average gain 8–16 wk. of the D_c group, compared to the D and D_t groups, was significantly different, ($P < .05$). This difference was also seen in the withers height gains (Table 4) and in dry feed intake (Table 4). The over-all poorer average performance of the calves in the D_c group, therefore, was apparently the combined result of lower feed intake and limited protein intake (Table 4). That the primary deterrent on growth may have been the low protein intake is also suggested by the relatively wide nutritive ratio of the D_c ration (1:6.96) (Table 1).

It was also considered significant that the calves in the D_t group performed as well as, and by some measurements better than, the calves in the D group, despite the replacement of part of the alfalfa hay in the pellets with timothy hay (Table 4). Although the D_t ration had a lower per cent protein and a lower dry matter and cellulose digestibility (Table 1) than the other two rations, protein digestibility was higher and the nutritive ratio was the narrowest of the three rations (1:5.09) (Table 1).

From the standpoint of efficiency of feed utilization, it was of interest that, measured in both weight of pellets per pound of gain and in TDN per pound of gain to 16 wk. of age, the Holstein calves were more efficient (Table 4). This was reflected in the growth curve of the Holsteins compared to the Jerseys (Figure 3). The D_t pellets were utilized more efficiently than the others on the basis of TDN per pound gain.

These results suggest that more work should be done on the relative merits of alfalfa and good mixed hay as the roughage component of high roughage pellets. Also, in order that protein intake will not be a limiting factor in growth, the data indicate that the total protein content of high roughage pellets should be at least 14.5% when alfalfa hay is used. Further work will be needed to determine whether or not the protein requirement can be supplied by the roughage without using a protein supplement.

Rumen-inoculated vs. uninoculated calves, Experiments 1, 2, and 3. In these experiments, as well as in those previously reported *(4, 5, 6, 14, 15, 17)*, in which calves were fed high roughage diets, rumen inoculations using fresh, warm cud material taken from older cattle were given weekly from 2 to 6 wk. of age. This practice was based on even earlier work *(20, 21)*, which showed that

by inoculating the calves the establishment of protozoa and certain indicator bacteria, usually seen in the rumens of adult cattle fed alfalfa hay and grain, could be accomplished earlier than by natural means. Further support for inoculating calves was the finding that, especially during the early weeks, the inoculated calves digested somewhat more cellulose *(9)* and more protein *(4, 9)* from their feed, had a different proportion of volatile fatty acids in their rumen juice *(12)*, and synthesized more thiamine, as indicated by increased urinary excretion *(8)*. These observations have served as the basis for recommending rumen inoculations in the high roughage system. In none of our previous experiments have sufficient numbers of calves been used to determine if growth was affected by rumen inoculations; thus, inoculations have never been used on the basis that growth would be enhanced.

Examination of Table 5 and Figure 4 shows that, whereas the average gains in body weight and withers height of the uninoculated calves were somewhat greater, the differences did not approach statistical significance. Further breakdown of the growth data according to the various pellet groups is shown (Table 6). Of particular interest in this connection was the observation *(7)* that even though the Hay II group of indicator rumen microorganisms was present in the cud material used for the inoculations, these becteria did not become established in the calves fed the pelleted rations, as they did when loose hay and grain were fed *(20, 21)*.

Differences in feed intake (Table 5 and Figure 5) and efficiency of feed utilization, measured as TDN per pound gain or weight of pellets per pound gain (Table 5), were not statistically significant, although the average efficiency of the uninoculated calves was somewhat greater. Average digestibility data (Table 5) showed that per cent digestibility of feed dry matter, cellulose, and protein was in favor of the inoculated calves, although the differences were not statistically significant.

On the basis of the measured differences between inoculated and uninoculated calves in these experiments, including the results reported in the companion paper *(7)*, showing that some of the bacteria did not become established, the practice of cud-inoculating calves fed high roughage pellets can not be justified. Further work will need to be done to determine whether the effects of inoculations may be expressed in improved growth and performance when less grain concentrates or poorer-quality hay is fed than was used in these experiments. Some evidence now available suggests that inoculations may be indicated when changing from a high grain ration to a high roughage ration *(20, 21)* or when discontinuing antibiotic feeding *(14, 19)*. Experiments reported by others showed that growth was not improved when calves were inoculated and fed various other unpelleted rations *(11)*. Therefore it is concluded, that, while rumen inoculations may be indicated under certain conditions, routine inoculations are not required for good growth in the high roughage system, where good hay is fed in a 2:1 ratio with a simple grain mixture, with milk to 7 wk. of age. The experimental results indicate that the protein content of the simple grain mixture should be adjusted so that the crude protein content of the entire ration is not

less than 14.5%. It is further suggested that 1% bone meal, dicalcium phosphate, or some other good source of phosphorus be included in the grain mixture.

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HIGH ROUGHAGE SYSTEM FOR RAISING CALVES BASED ON EARLY DEVELOPMENT OF RUMEN FUNCTION. IX. EFFECT OF RUMEN INOCULATIONS AND CHLORTETRACYCLINE ON RUMEN FUNCTION OF CALVES FED HIGH ROUGHAGE PELLETS

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SUMMARY

Early rumen function was studied in 71 Jersey calves fed pelleted high roughage mixtures (two parts hay to one part grain) from three days to 16 wk. of age. The establishment of rumen-indicator microorganisms, total steam volatile fatty acids in the rumen, rumen pH, and in vitro cellulose digestion were used as measures of rumen development. The effects of rumen (cud) inoculations, chlortetracycline, and supplementing high roughage pellets with a combination of molasses, monocalcium phosphate, distillers solubles, and brewers yeast were also studied by employing a factorial type of experimental design.

In all calves fed high roughage pellets, the development of rumen function was characterized by a rapid rise in rumen volatile fatty acids to a maximum at 6 wk. of age, relatively low rumen pH values, the absence of Hay II indicator microorganisms, and a gradual increase in the ability of rumen contents to digest cellulose with advancing age of the calf in vitro. No important differences affecting early rumen fermentation were noted from feeding chlortetracycline; a molasses, calcium monophosphate, distillers solubles, and brewers yeast supplement, or different levels and sources of protein. However, in vitro tests showed that including chlortetracycline in the basal high roughage pellet reduced propionic acid yield.

Feeding either chlortetracycline- or molasses-containing supplements to calves suppressed in vitro cellulose digestion slightly. This difference was more marked when the supplemented pellets were digested in vitro with a standard inoculum.

Rumen-inoculated calves were observed to have high average ratings for rumen protozoa, but nearly all of the uninoculated calves remained free of rumen protozoa until 16 wk. of age. Inoculations also increased the average rating of Hay I indicator microorganisms and the in vitro digestion of cellulose by rumen microorganisms. Inoculating resulted in higher rumen levels of butyric acid and lower propionic acid, but reduced the rumen hydrogen ion concentration during the age period of 2–5 wk.

In the three experiments herein reported, various measures of rumen function were made, using calves fed different batches of high roughage pellets (two parts hay to one part grain). Data on growth, feed consumption, and utilization were presented in the previous paper *(13)*, in which it was concluded that pelleting high roughage mixtures for young calves stimulated early dry feed consumption, prevented selective eating, and resulted in increased growth. In earlier experiments *(13, 15, 16, 24, 25)* involving high roughage feeding of calves, it was observed that normal rumen function characteristic of mature cows developed after calves had eaten appreciable amounts of dry feed for a period of 2–3 wk.

During the course of experiments leading to the development of the high roughage system for feeding calves *(16)*, rumen (cud) inoculations were used on the basis that this procedure was a satisfactory method of early establish-

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ment of many kinds of rumen microorganisms, including both protozoa and bacteria, usually found in the rumens of mature cows *(24)*. It was hypothesized that the early establishment of mature-ruminant types of microorganisms would enhance rumen digestion and synthesis when high roughage rations were fed. The over-all results of experiments carried out to date show greater cellulose and protein digestibility, changes in proportion of rumen volatile fatty acids, and increased thiamine synthesis in the rumens of inoculated calves *(8, 9, 10)*. However, as shown in the previous report *(26)* and in the companion paper *(13)*, cud inoculations have not resulted in increased growth or better performance when a 2:1 ratio of hay to grain was fed, using good quality hay.

In this study, an effort has been made to determine the over-all effects of feeding high roughage pellets on the establishment of certain rumen indicator microorganisms, total steam volatile acids in the rumen, partition of rumen volatile fatty acids, rumen hydrogen ion concentration, and the in vitro digestion of cellulose and volatile fatty acid production in calves. In conjunction with this study the effects of rumen inoculations, addition of chlortetracycline and mineral and vitamin supplements, and variations in the level and source of protein were observed.

EXPERIMENTAL PROCEDURES

In three experiments 71 Jersey calves, all of which are included in the data presented in the companion paper *(13)*, have been used as the source of rumen material in this study. The pelleted high roughage rations also have been described, along with calf performance. All pellets (¼-in. diameter) consisted of two parts hay (ground through a ½-in. hammer mill screen and one part grain concentrate). The ingredients used in formulating the eight kinds of pelleted rations are shown in Table 1. of the companion paper *(13)*. The pellets were offered free choice after the third day and constituted the only feed during the period of the 7th to the 16th wk.

Rumen samples containing from 50 to 75 ml. of rumen contents were taken by stomach tube at weekly intervals until 8 wk. of age, and biweekly from 8 to 16 wk. Sampling was done 3 hr. after A.M. feeding. Immediately after the sample was taken, the pH was determined using a Beckman Model G pH meter. Wet preparations for protozoa and dry smears for Gram stains were made on slides for ratings of rumen microorganisms, using procedures previously described *(24)*. The samples were taken to the laboratory in 75-ml. test tubes immersed in a warm water bath, strained through a double thickness of 90-gauge cheesecloth, and pressed out. The strained rumen juice was used for in vitro fermentation studies and volatile fatty acid analyses. An artificial rumen procedure was used to follow early development of rumen function in a manner similar to that employed by McCarthy and Kesler *(22)*. However, in this study the basal pelleted high roughage rations (A and D) were ground through a Wiley mill and used as the substrate with the mineral buffer mixture of Bentley *et al. (4)*. Fermentations were carried out for a period of 21 hr. in Experiment 1 and 2, and for 12 hr. in Experiment 3. Cellulose was determined by the method of Crampton and Maynard *(11)*. Total steam volatile fatty acids were deter-

mined and chromatographic separations were made to determine the proportions of acetic, propionic, and butyric (including some higher acids) acid in the rumen juice. The chromatographic separations were made by the method of Bulen *et al. (6)* as adapted to rumen juice by Linke *(19)*.

In Experiment 1 the effects of adding chlortetracycline, vitamin and mineral supplements, and rumen inoculations were studied with hay made from the same crop of alfalfa for all rations. Two groups of ten calves each were fed pelleted high roughage rations A or B. The basal A group was fed A pellets to 16 wk. The B pellets, which contained 20 mg. of chlortetracycline per pound, were fed to the B group to 12 wk. After 12 wk., the B group was switched to A pellets and continued to 16 wk. Half of the calves in both A and B groups were given cud inoculations at 2, 3, 4, 5, and 6 wk. of age. In order to test whether or not added vitamins and minerals would encourage early rumen function, five uninoculated calves were fed C pellets to 8 wk. of age. The C pellets contained molasses, monocalcium phosphate, distillers solubles, and brewers yeast as supplements.

In Experiment 2, more observations were made on the effect of rumen inoculations with and without vitamin and mineral supplements using C_1 pellets made from a different batch of alfalfa than was used in Experiment 1. Ten calves, C_1 group, received C_1 pellets to 12 wk. of age and loose alfalfa hay and grain (14.5% protein) fed in a 2:1 ratio from 12 to 16 wk. of age. During the first 12 wk. they were compared to six calves receiving the basal A_1 pellets. One-half of the calves in each group were cud-inoculated at 2, 3, 4, 5, and 6 wk. of age.

Experiment 3 was designed to study the effects of reducing the total protein in the basal D pellets from 14.3 to 12.3% by two methods: *(1)* In the D_c pellets by replacing all the soybean meal with ground corn and *(2)* in the D_t pellets by replacing half the alfalfa hay with timothy hay. Because of mechanical difficulties in the mixing process, less timothy hay and less grain were put into the D_t pellets than was anticipated. The protein content of the resulting D_t pellets was reduced to 13.4% only. Ten calves were used in each group, one-half of which were rumen-inoculated at 2, 3, 4, 5, and 6 wk. of age.

For purposes of reference the experimental groupings in all three experiments are presented in Table 2 of the companion paper *(13)*, except that the C_2 and C_3 group were not used.

RESULTS AND DISCUSSIONS

The average ratings of indicator rumen microorganisms in rumen contents of calves fed eight kinds of high roughage pellets are presented (Table 2). These data show that no essential differences were found among the various pellet groups in the numerical ratings for rumen microorganisms, regardless of the supplemental feeds used. It is noteworthy that the Hay II microorganisms *(24)* failed to become established, even though these microorganisms were present in the cud material used. Table 1 shows the average rating of rumen microorganisms in the material used for rumen inoculations. Comparison of results in

TABLE 1

Average rating of indicator rumen microorganisms[a] in cud material from older cows used for rumen-inoculating calves

Experiments	No. of samples	Indicator microorganisms		
		Protozoa[b]	Hay I[c]	Hay II[d]
Experiment 1	6	4	3.0	7.0
Experiment 2	2	4	4.0	7.0
Experiment 3	4	4	3.5	3.8

[a] Indicator microorganisms and rating method previously described *(24)*.
[b] Protozoa rated 0-4, using wet preparations.
[c] Hay I—large G+ coccoids in closely knit pairs, rated 0 to 4.
[d] Hay II—three bacteria, large G+ square-ended rods, very large G− cigar-shaped rods, G− short rods in fours and multiples of four, rated individually 0 to 4, and summed.

Tables 1 and 2 reveal that cud-inoculated calves fed high roughage pellets had ratings of the indicator rumen microorganisms at 6–8 wk. of age, similar to those found in older cattle, except that the Hay II microorganisms did not become established.

It is interesting that Hay II microorganisms absent from the calves in these experiments were also found to be absent from the rumens of calves fed high grain rations *(15, 25)* or chlortetracycline *(14)*, and in uninoculated calves isolated from older cattle *(24)*. Thus, the differences in Hay II microorganisms observed previously *(14)* from feeding chlortetracycline could not be followed in Experiment I. Other workers *(17, 20, 21)* have observed an effect of chlortetracycline on rumen microorganisms. Mann *et al. (21)* showed that chlortetracycline-fed calves had larger numbers of rumen protozoa at an early age than untreated calves. This increase was attributed to increased roughage intake. The results presented (Table 2) for the B pellet group suggest that this may have been true in our experiments but they are not conclusive on this point.

The marked differences in the establishment of indicator rumen microorganisms (shown in Tables 1 and 2) reaffirm earlier work *(24, 25)*. Rumen-inoculated calves had relatively high ratings for protozoa at 6 wk. of age. Nearly all of the uninoculated calves remained free of the usual rumen protozoa and consequently depended entirely on bacteria for rumen digestion and synthesis. Bryant and Small *(5)* have also observed that many kinds of bacteria and the full complement of protozoa that predominate in mature cattle became established in inoculated calves by 6–9 wk. of age. Whereas few of these bacteria and none of the protozoa were found in isolated uninoculated calves, the total bacteria counts exceeded ten times the number in inoculated calves. Furthermore, they noted that propionic acid bacteria were especially prevalent in the isolated calves at an early age, but were replaced with other kinds as the calves advanced in age.

Cud-inoculated calves had higher ratings for Hay I microorganisms. The differences in Hay I microorganisms were more marked at 8 than at 16 wk. of age. This confirms earlier experiments *(10, 24)* which indicated that Hay I microorganisms gradually became established in uninoculated calves, although at a slower rate.

TABLE 2

Average rating of indicator rumen microorganisms[a] in rumen contents from cud-inoculated and uninoculated Jersey calves fed high roughage pellets

Indicator microorganisms	Protozoa[b]							Hay I bacteria[c]		Hay II bacteria[d]	
Week of age	1	3	4	6	8	12	16	8	16	8	16
A Pellets											
Inoculated	0	1.6	1.4	3.0	3.0	3.4	3.6	2.4	2.2	0	0
Uninoculated	0	0	0	0	0	.8	.8	1.0	2.0	0	0
B Pellets											
Inoculated	0.6	1.2	2.2	3.4	3.4	4.0	3.9	2.0	3.0	0	0
Uninoculated	0	0	0	0	0	0	0	0.7	1.8	0	0
C Pellets											
Uninoculated	0	0	0	0	0	0.4	1.2	1.0	2.0	0	0
A_1 Pellets											
Inoculated	0	1.2	2.1	2.6	3.0	4.0	–	1.1	–	0	–
Uninoculated	0	0	0	0	0	0	–	1.0	–	0	–
C_1 Pellets											
Inoculated	0	2.6	2.6	3.2	4.0	3.5	4.0	3.0	1.2	0.8	0
Uninoculated	0	0	0	0	1.2	2.0	2.4	2.0	2.3	0	0.8
D Pellets											
Inoculated	0	0.2	1.2	2.3	3.3	3.5	4.0	4.0	4.0	0	1.0
Uninoculated	0	0	0	0	0	0	0	0.7	1.5	0	0
D_t Pellets											
Inoculated	0	1.6	2.0	3.0	3.4	3.6	3.6	3.0	3.3	0	0.5
Uninoculated	0	0	0	0	0	0	0	2.0	3.3	0	0
D_c Pellets											
Inoculated	0	1.0	2.3	2.2	2.6	3.6	3.9	4.0	3.0	0	0.5
Uninoculated	0	0	0	0	0	0	0	1.0	2.5	0	0

[a] Indicator microorganisms and rating method previously described *(24)*.

[b] Protozoa rated 0-4, using wet preparations.

[c] Hay I—large G+ coccoid in closely knit pairs, rated 0 to 4.

[d] Hay II—three bacteria, large G+ square-ended rods, very large G− cigar-shaped rods, G− short rods in fours and multiples of four, rated individually 0-4, and summed.

TABLE 3

Average pH[a] of rumen contents from calves fed high roughage pellets containing various supplements

	Experiment 1			Experiment 2		Experiment 3		
Pellet group	A	B	C[b]	A_1	C_1	D	D_t	D_c
No. of calves	10	10	5	6	10	10	10	10
Age at sampling *(wk.)*								
1	6.44	6.53	6.32	6.53	6.52	6.14	6.46	6.32
2	6.45	6.38	6.27	6.22	6.08	.....	.....	.....
3	6.19	6.13	5.89	5.93	5.52	5.89	6.01	5.95
4	5.92	5.98	5.95	5.64	5.68	5.93	6.39	5.88
5	6.13	6.08	5.95	5.70	5.75	.....	.....	.....
6	6.22	6.12	5.88	6.05	5.85	5.93	6.04	6.12
7	6.42	6.15	6.07	6.09	5.95	.....	.....	.....
8	6.13	6.35	6.05	6.32	6.17	6.12	6.25	6.41
10	6.54	6.28	6.28	.....	.....	.....	.....	.....
12	6.45	6.49	6.58	6.68	6.04	6.16	6.19	6.15
14	6.56	6.52[c]	6.20	.....	.....	.....	.....	.....
16	6.58	6.38[c]	6.70	.....	6.50[d]	6.16	6.26	6.43

[a] The range in pH readings was 5.22–7.20.

[b] All the calves in this group were uninoculated. One-half of the calves in each of the other groups were cud-inoculated.

[c] Fed A pellets after 12 wk. of age.

[d] Fed loose hay and grain in a 2:1 ratio from 12–16 wk.

The changes in average total rumen volatile fatty acids with increasing age are shown (Figure 1). A comparsion of total rumen volatile fatty acids and

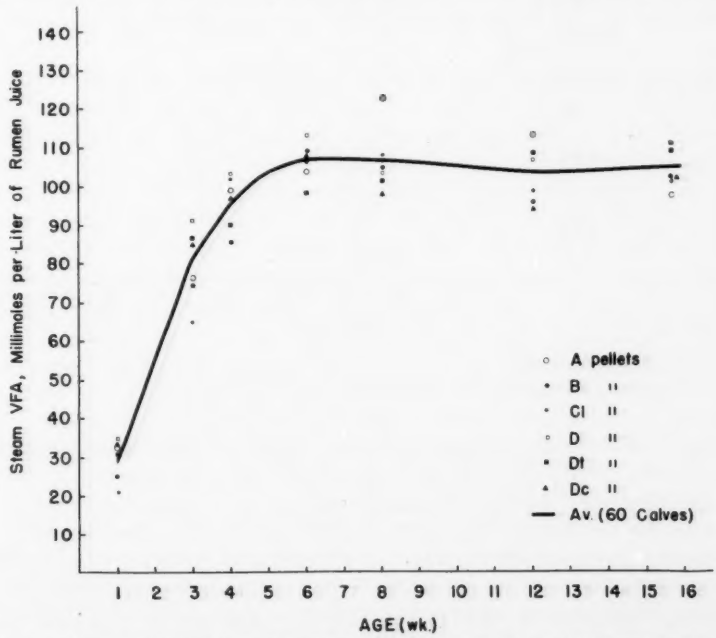

FIG. 1. Steam volatile acid in the rumen juice of calves fed six different pelleted high roughage rations for a period of 16 wk.

pH in inoculated and uninoculated calves from Experiment 1 is presented (Figure 2). The average pH values of rumen contents from calves fed different kinds of pellets are given (Table 3). The average content of total steam volatile fatty acids in the rumen juice was strikingly similar for all pellet groups as well as for the cud-inoculated and uninoculated calves (Figures 1 and 2). The level of volatile fatty acids increased sharply after the first week as the calves

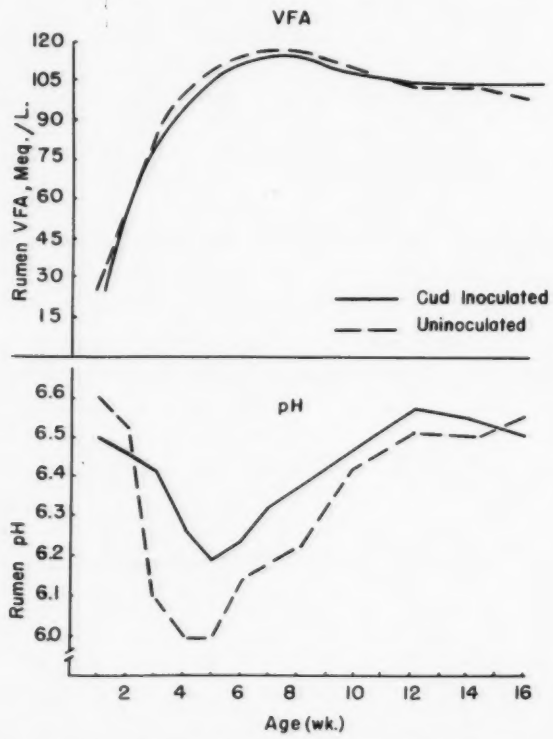

FIG. 2. The steam volatile fatty acid and pH of rumen juice of ten rumen-inoculated and ten uninoculated calves fed high roughage pellets in Experiment 1.

commenced eating high roughage pellets and reached the maximum range at 6 wk. of age. It will be noted that the line graph representing the average levels for all groups declined 4 to 5 milliequivalents from the maximum level at 6 and 8 wk. as calves grew older. A similar curve for total rumen volatile fatty acids is shown in the results of McCarthy and Kesler *(22)*. In contrast to these results, our previous experiments with chopped clover–timothy hay gave maximum values at 9 wk. of age *(15)*.

It is interesting that the rumen pH dropped to relatively low values during the first 5 wk. of age while rumen volatile fatty acid levels were increasing at

the greatest rate. They then reversed and gradually reached levels characteristic of the rations by 8 to 10 wk. of age. As in other experiments *(15)*, these results suggest that young calves were unable to buffer sufficiently to offset rumen fermentation. There exists also the possibility that the calves were less able to absorb volatile fatty acids at this early age. Proper development of the rumen papillae which provide the major areas of absorption in this organ depends on the presence of volatile fatty acids *(12)*.

Among the various pellet groups, three significant differences occurred in rumen pH during the first 5 wk. of age. First, the data in Table 5 show a definite trend toward lower rumen pH values when the molasses-supplemented C and C_1 pellets were fed. This was particularly true at 2 and 3 wk. of age, when the calves exhibited limited ruminal buffering capacity. Secondly, the pH readings were higher in Experiment 1 at 2, 3, 4, and 5 wk. of age than in Experiment 2 ($P < .01$). This suggests that less buffering capacity resulted from the hay used in the latter experiment. Finally, in Experiment 3, the rumen pH in calves fed D_t pellets which contained approximately one-half timothy hay was significantly higher ($P < .01$) at 3 and 4 wk. of age than in calves receiving the basal D pellets.

These results clearly indicate that rumen fermentation was increased at an early age in calves fed pelleted high roughage mixtures. This effect may be attributed to increased feed intake *(13)* and increased concentration of dry matter in the rumen contents, as observed in experiments by Balch *(3)* using finely ground hay.

The average molar percentages of acetic, propionic, and butyric acids (including some higher acids) in the rumen juice of cud-inoculated calves fed the various kinds of high roughage pellets are presented (Table 4). No important differences occurred among pellet groups within any of three experiments, but the proportion of volatile fatty acids varied among experiments in which different sources of alfalfa hay were used. Acetic acid was lower and butyric acid was higher in Experiment 1 compared to both Experiments 2 and 3, which were similar. These data indicate that either the hay and/or protein content of the high-roughage pellets effected a shift in proportion of volatile fatty acids.

More striking differences in the volatile fatty acids were found between cud-inoculated and uninoculated calves. It will be noted that the proportion of propionic acid was higher in all groups of uninoculated calves. Also, that the proportion of propionic acid in rumen juice of uninoculated calves decreased with age approximating the amount in the rumen of inoculated calves at 16 wk. These differences were highly significant ($P < .01$). In contrast, an over-all summary showed that the proportion of acetic acid tended to increase with age in uninoculated calves, but within groups this increase with age was not consistent. Butyric acid remained approximately the same at all age groups in the rumen juice of both cud-inoculated and uninoculated calves, despite the random occurrence of marked variations among week-groups. The proportion of butyric acid was significantly higher in the rumen of cud-inoculated calves ($P < .01$) in Experiment 1. Butyric acid also tended to be higher in cud- inocu-

TABLE 4

Average molar percentages of acetic, propionic, and butyric acids in the rumen juice of cud-inoculated and uninoculated Jersey calves fed high roughage pellets

Group	Experiment 1				Experiment 2				Experiment 3					
	A		B[a]		A_1		C_1[b]		D		D_t		D_c	
No.	Inoculated 5	Uninoculated 5	Inoculated 5	Uninoculated 5	Inoculated 3	Uninoculated 3	Inoculated 5	Uninoculated 5	Inoculated 4	Uninoculated 5	Inoculated 5	Uninoculated 5	Inoculated 5	Uninoculated 5
Age. *(wk.)*						Acetic Acid *(mole %)*								
4	.....	.....	.....	.....	66.9	68.0	62.3	66.5	62.9	60.5	63.4	69.1	64.6	57.4
6	64.3	55.1	58.8	63.5	63.9	62.4	68.8	63.0	75.4	65.8	65.3	65.2	67.4	57.7
8	63.1	62.8	62.9	60.9	67.1	63.8	72.9	69.6	68.2	63.1	67.6	65.2	67.3	60.8
12	65.1	64.8	62.4	60.8	70.4	67.8	72.2	72.8	74.3	66.7	71.1	65.6	69.5	69.7
16	65.6	62.7	64.5	64.4	.....	.....	74.6	71.2	77.7	68.5	65.2	67.2	69.2	69.4
						Propionic Acid*(mole %)*								
4	.....	.....	.....	.....	21.1	25.1	25.9	25.0	21.0	27.6	23.2	24.5	25.4	30.6
6	19.5	31.6	20.9	25.0	20.8	30.2	18.8	27.6	21.5	27.2	19.1	28.1	23.2	30.8
8	17.0	23.1	19.6	26.1	22.0	27.5	16.7	20.8	20.0	28.1	18.2	24.6	22.2	32.9
12	17.6	23.9	19.2	25.1	18.7	22.9	17.6	17.8	18.9	25.2	16.8	30.7	20.3	23.7
16	17.6	23.3	18.9	21.6	.....	.....	17.7	19.8	17.7	24.1	22.2	25.1	17.4	21.4
						Butyric Acid[c] *(mole %)*								
4	.....	.....	.....	.....	12.0	6.9	11.8	8.6	7.7	8.2	10.1	6.6	10.0	12.1
6	15.8	13.3	20.3	11.5	15.3	7.3	10.9	9.5	11.5	7.3	6.2	6.7	9.4	11.3
8	19.9	14.1	17.6	13.0	10.8	8.8	10.5	9.3	11.8	11.2	12.1	10.3	10.6	6.4
12	17.1	11.1	18.4	14.1	10.9	9.3	10.1	9.4	9.2	8.7	10.0	11.6	10.2	6.5
16	16.8	11.2	15.6	13.2	.....	.....	7.7	9.0	10.0	11.7	12.6	6.8	13.5	9.1

[a] B pellets were replaced with A pellets at 12 wk.; thus, no chlortetracycline was fed 12–16 wk.
[b] Changed to loose hay and grain, 2:1 ratio, at 12 wk.
[c] Includes some higher acids.

lated calves in Experiments 2 and 3, but the differences were not consistent among groups.

That these differences in proportions of rumen volatile fatty acids between cud-inoculated and uninoculated calves reflected quantitative differences in grams per liter of rumen content is shown (Figure 3) by comparison of data from ten cud-inoculated and ten uninoculated calves. The prevalence of propionic acid bacteria in the uninoculated calves of Bryant and Small *(5)* and their subsequent decrease follows approximately the same age pattern as the peak level

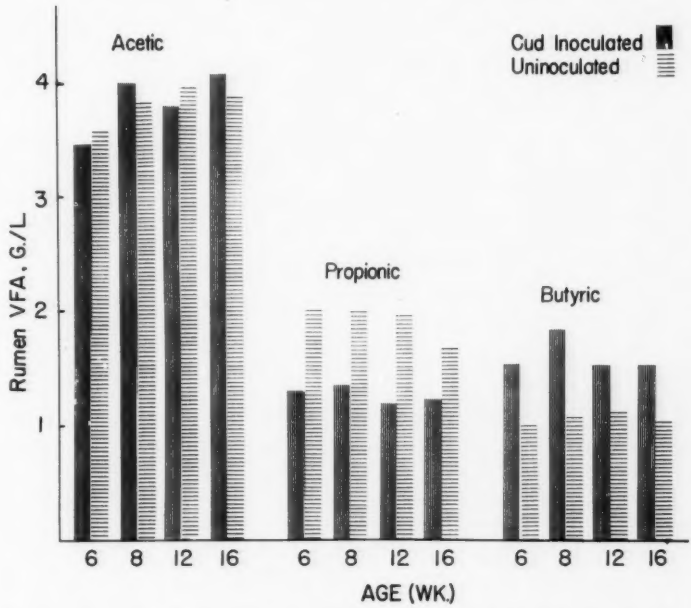

FIG. 3. Changes in the amount of acetic, propionic, and butyric acid in the rumen juice of rumen-inoculated and uninoculated calves at 6, 8, 12, and 16 wk. of age.

of propionic acid and its subsequent decline with age observed in the uninoculated calves of this experiment (Table 4 and Figure 3). This is also in accord with the results of Lengemann and Allen *(18)*, which showed that more propionic and less acetic acid were present in the rumen at 4 and 8 wk. of age than in calves 6 mo. of age fed the same ration of milk, grain, and hay.

Increasing ability of rumen microorganisms from calves to digest cellulose with increasing age of animals is shown in Table 5, which gives the in vitro volatile fatty acid production and cellulose digestion when inoculum from the inoculated and uninoculated calves fed high roughage pellets was used. Feeding chlortetracycline in Experiment 1 and the substitution of molasses, monocalcium

TABLE 5

In vitro steam volatile fatty acid production and cellulose digestion, using as the inoculum rumen microorganisms from cud-inoculated and uninoculated calves fed high roughage pellets

Experiment	Experiment 1					Experiment 2				Experiment 3	
Pellet group	A		B[a]		C	A_1		C_1[b]		D, D_1, D[c] combined	
Rumen inoculation	Inoculated	Uninoculated	Inoculated	Uninoculated	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated	Inoculated	Uninoculated
No. of calves	5	5	5	5	5	3	3	5	5	14	15
Hr. fermented	21	21	21	21	21	21	21	21	21	12	12
Age *(wk.)*	Volatile fatty acids produced,[c] *(meq/g of feed)*										
1	3.6	3.6	3.1	2.6	3.3	2.4	2.1	2.5	2.6	1.8	2.0
3	4.4	4.4	4.5	3.9	4.1	3.0	2.7	2.9	2.8	2.4	2.3
4	4.7	4.7	4.1	4.1	4.1	4.0	3.5	3.3	3.2	2.5	2.7
6	4.4	4.7	4.9	4.4	4.5	4.3	3.6	4.1	3.8	2.9	2.5
8	5.3	4.9	4.6	4.4	4.8	3.9	4.1	4.2	3.8	3.2	2.2
12	4.7	4.8	4.4	4.3	4.6	4.0	3.4	4.1	4.0	3.1	2.5
16	4.8	4.4	4.2	4.6	4.1	....	.....	4.1	4.2	2.5	2.5
	Percentage cellulose digested[d]										
1	21.4	19.8	15.7	10.7	6.7	8.3	6.0	12.8	10.8	5.7	14.6
3	30.0	23.2	15.8	20.9	29.6	15.4	12.7	12.6	13.5	10.1	12.3
4	25.3	19.0	24.5	22.7	25.5	27.7	19.2	18.6	10.6	16.0	9.1
6	29.3	30.8	29.8	19.3	27.4	30.4	17.1	28.2	13.0	16.6	9.2
8	37.1	33.1	30.2	24.1	27.3	30.0	24.1	28.6	21.6	24.7	13.0
12	37.0	36.0	35.0	30.4	39.4	28.8	23.6	29.9	22.3	27.1	16.9
16	40.0	37.5	32.6	39.7	37.5	.....	.....	26.4	27.4	21.5	18.1

[a] B pellets were replaced with A pellets at 12 wk. of age; thus, no chlortetracycline was fed 12–16 wk.

[b] Changed to loose hay and grain 2: 1 ratio, at 12 wk.

[c] Differences between means in Experiment 2 statistically significant ($P < .01$). Standard deviation for individuals within all groups was $\pm$ 0.36.

[d] Differences between means in Experiments 2 and 3 statistically significant ($P < .01$). Standard deviation for individuals within all groups was $\pm$ 7.7.

phosphate, distillers solubles, and brewers yeast for portions of the ground corn and soybean oil meal in Experiment 1 and 2 limited in vitro cellulose digestion, but not significantly. In these experiments, the basal A and A_1 pellets were used as the substrate. Using a standard inoculum, the suppression of cellulose digestion was more marked when the digestion of the basal A pellets was compared to B pellets which contained chlortetracycline and C pellets which contained molasses and other supplements (Table 6). These findings are in agree-

TABLE 6

The in vitro production of volatile fatty acids and cellulose digested using a standard inoculum and finely ground high roughage pellets as the substrate (Experiment 1)

	Volatile fatty acids				Cellulose digested
	Total	Acetic	Propionic	Butyric	
	meq/21 hr.	(mg/21 hr.)			
A pellets	4.4	114.0	65.7	52.9	60.1
B pellets[a]	3.8	87.9	26.4	47.4	33.2
C pellets[b]	4.1	95.8	45.5	31.7	43.0

[a] 20 mg. of chlortetracycline per pound of pellets.
[b] Supplements contained in C pellets included distillers solubles, molasses, monocalcium phosphate, and brewers yeast.

ment with results of other workers *(2, 22)*. No differences were observed in the in vitro digestion among the groups fed different levels of protein in Experiment 3. Consequently, the results were combined to simplify presentation (Table 5).

Rumen-inoculating calves improved in vitro cellulose digestion in all experiments. By reducing fermentation time from the 21-hr. period used in Experiments 1 and 2, to 12 hr. in Experiment 3, the differences between cud-inoculated and uninoculated calves were accentuated, again demonstrating less cellulose digestion in uninoculated calves. The differences diminished as the calves grew older. This confirms in vivo observations *(10)*. A part of the increased cellulose digestion in the rumen of inoculated calves may result from protozoan action which is absent in uninoculated calves. Oxford *(23)* has shown that protozoa are capable of appreciable cellulose digestion in the presence of rumen bacteria.

Differences in volatile fatty acids produced tended to follow cellulose digestion, but were less marked.

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INFLUENCE OF ANISE OILS ON THE PALATABILITY OF CALF STARTERS[1]

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SUMMARY

Two imitation anise oils and U.S.P. anise oil were used in cafeteria experiments which involved a wide range of basal starters. In each of four experiments, anise oil significantly depressed palatability. Anise oil was more detrimental in one experiment than in the others. In one experiment U.S.P. anise oil and one of the imitations lowered palatability highly significantly more than did another imitation. Two pounds of imitation anise oil per ton of starter lowered palatability highly significantly more than did one-half pound per ton.

Anise oil and various imitations have been widely used in feeds, with the objective of improving their palatability. However, there is a dearth of published research data concerning the effect of anise oil on the palatability of feeds, including calf starters. Since anise oils are expensive, it seemed desirable to determine whether they improved palatability and, if there was an improvement, to determine the most desirable level of anise oil to use.

EXPERIMENTAL PROCEDURE

This study was composed of four cafeteria-type experiments, the last two of which were performed simultaneously. The two imitation anise oils were arbitrarily designated as No. 1 and No. 2 for convenience in presenting the data. Imitation anise oil No. 1 was used in all of the experiments. In the fourth experiment, imitation anise oil No. 2 and U.S.P. anise oils were used in comparison. The primary ingredients used in the basal starters varied widely among the experiments. Composition of the basal starters is presented (Table 1).

In the first experiment, three calves were offered starters with 0, 0.5, or 2.0 lb. of imitation anise oil No. 1 per ton of feed for a 10-wk. period. Each level was fed with soybean oil meal and with degossypolized cottonseed oil meal.

In the second experiment, seven calves were offered starter containing 0 and 0.5 lb. of imitation anise oil No. 1 per ton of starter. Each level was offered with soybean oil meal and with degossypolized cottonseed oil meal. The length of time which these calves were used in the experiment varied from 1 to 7 wk. and averaged 3 wk. They were on experiment for the following weeks of age dating from the sixth day of age: *(a)* 8th wk.; *(b)* 7th wk.; *(c* and *d)* 7th and 8th wk.; *(e)* 7th through 9th wk.; *(f)* 6th through 9th wk., and *(g)* 5th through 11th wk.

The third and fourth experiments were conducted on alternate weeks with the same group of ten calves used for 10 wk. In the third experiment, the calves

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TABLE 1

Basal starters used in each of the four experiments

Ingredient[a]	Experiment No. 1	2	3	4
	(lb 100 lb. starter)			
Cottonseed oil meal (degossypolized)	(27)[b]	(35)[b]	....	....
Corn, ground	43	30	....	....
Oats, ground	22	....	....	....
Soybean oil meal (solvent extracted)	(27)[b]	(35)[b]	32	32
Citrus pulp	....	31	64	60
Brown grease	2	....	....	2
Hydrogenated cottonseed oil	2	....	....	2
Supplements[a]	4	4	4	4

[a] All of the starters contained the following per 100 lb. of starters: *1)* one lb. Aurofac 2-A (3.6 g. chlortetracycline hydrochloride per lb.) (American Cyanamid); *2)* one lb. defluorinated rock phosphate (Coronet); *3)* one lb. trace-mineralized salt with the following guaranteed analysis (NaCl, 96.9–99.2%; Mn 0.25%; Fe, 0.16%; Cu, 0.033%; Co, 0.010%; I, 0.007%; and Zn, 0.005%); *4)* 200 g. vitamin A supplement (Dawe's Fixtay–20,000 USP units/gm), and *5)* 200 g. vitamin D supplement (Dawe's Sterol D_3, 1,500 I.U/g in soybean oil meal).

[b] Only one of these oil meals was used in any one starter, but the experiments contained starters with each.

were given a choice between starters containing 0.5 lb. of imitation anise oil No. 1 per ton and no anise oil. The basal starter contained only citrus pulp and soybean oil meal plus supplements (Table 1). For the fourth experiment, 2% brown grease and 2% hydrogenated cottonseed oil replaced like amounts of citrus pulp. In other respects, the basals were identical (Table 1). The effects of no anise oil and 0.5 lb. per ton of U.S.P. anise oil, imitation anise oil No. 1, and imitation anise oil No. 2 were compared. Each of the imitations are regarded by the manufacturers as being equivalent to the U.S.P. product on an equal-weight basis for flavoring feeds.

The calves stayed with their dams for the first day, then were taught to drink colostrum and milk from an open pail until six days old. The calves used in the first experiment were then given 252 lb. of a commercial milk replacer over a period of 6 wk. The replacer used was guaranteed to contain not less than 24% crude protein, 10% crude fat, and 50% nitrogen-free extract, and not more than 0.25% crude fiber. The principal ingredients were dried skimmilk, dried whey, stabilized animal fat, and lecithin. The replacer contained added vitamin A, D, and E, chlortetracycline, Fe, Mn, I, Cu, Co, and Zn. This product was mixed with nine parts water by weight to make up the replacer which was fed.

The calves in the second experiment were given an average of 193 lb. of whole milk and 199 lb. of milk replacer over a period averaging 51 days. The female calves in the third and fourth experiments were given equal amounts of whole milk and milk replacer for the first week of the experiment, and milk replacer for five additional weeks. The male calves received only milk replacer for the 6-wk. period. Holstein and Jersey females received 28 and 21 lb. of whole milk and 277 and 242 lb. of milk replacer, respectively. Holstein and Jersey males received 305 and 263 lb. of milk replacer, respectively.

Chopped alfalfa hay was fed ad libitum and water was offered twice daily.

Each calf was given more of each of the starters in the cafeterias than it would consume. At the end of each 24-hr. period the refusals were removed, weighed, and the positions of the feed boxes re-randomized. The amounts of each of the starters consumed were summarized on a weekly basis for statistical analyses.

The differences between treatments were so large in the first experiment that the sign-test method of statistical analysis, as given by Dixon and Massey *(2)*, was used. The results of the other experiments were converted to common logarithms to remove the nonhomogeneity of variance caused by calves eating much more as they became older. The transformed data was then used in the analysis of variance *(1)*.

RESULTS AND DISCUSSION

Total consumption of each starter for each of the calves employed in the first experiment is presented [Table (2a)]. The starter with no imitation anise oil and soybean oil meal averaged 78% of the total eaten. This was highly significantly more than was eaten of any other starter. Consumption of the starters with 0.5 lb. of the imitation anise oil No. 1 per ton was highly significantly higher than that of the starters with 2.0 lb. per ton. Since starters with higher levels of the imitation anise oil No. 1 were less palatable, only the lower level of anise oil was used in later experiments.

TABLE 2

Effect of anise oil on the palatability of calf starters in cafeteria experiments

Table 2a. *Effect of level of imitation anise oil No. 1 on starter consumption with two high protein sources*

Calf No.	Soybean oil meal *(lb. anise oil/ton)*			D.G.[a] cottonseed oil meal *(lb. anise oil/ton)*		
	0	0.5	2.0	0	0.5	2.0
	(lb. starter/wk)					
1	10.5	0.2	0.3	0.2	0.1	0.1
2	13.0	3.2	0.4	0.6	0.9	0.4
3	17.1	2.0	0.5	1.2	1.3	0.3
Av.	13.5	1.8	0.4	0.7	0.8	0.3

In the second experiment, citrus pulp was added to the basal starter, and the ground oats, brown grease, and hydrogenated cottonseed oil were removed (Table 1). The average results of this experiment are presented [Table (2b)]. The four starters employed and the average pounds of starter consumed per calf per week were as follows: *(a)* soybean oil meal, without anise oil, 8.8; *(b)* soybean oil meal, with imitation anise oil No. 1, 6.3; *(c)* degossypolized cottonseed oil meal, without anise oil, 4.8, and *(d)* degossypolized cottonseed oil meal with imitation anise oil No. 1, 3.3. The larger consumption of the starters without anise oil was significant at the 6% probability level. However, the detrimental effect of the imitation anise oil No. 1 was not nearly so large as that found in the earlier experiment.

Table 2b. *Effect of 0.5 lb/ton of imitation anise oil No. 1 on starter consumption with two high protein sources*

Calf	No. wk.	Soybean meal		D.G.[a] cottonseed oil meal	
		Control	Anise	Control	Anise
		(av. lb. starter/calf/wk)[b]			
a	1	5.4	2.4	4.8	1.8
b	1	9.0	0.0	3.6	0.5
c	2	7.4	1.8	14.4	5.8
d	2	2.6	1.5	15.2	6.0
e	3	8.6	8.9	8.8	6.0
f	4	9.5	7.9	1.2	1.4
g	7	13.1	14.2	3.7	3.7
	Av.[b]	8.8	6.3	4.8	3.3

Table 2c. *Effect of imitation anise oil No. 1 on starter consumption with high citrus pulp basal*

Calf No.	Control	Imitation anise oil No. 1
	(av. lb. starter/calf/wk)[b]	
1	4.2	4.9
2	5.1	2.1
3	7.3	5.7
4	5.1	5.6
5	2.8	1.9
6	5.9	5.6
7	9.2	9.3
8	5.1	2.7
9	8.5	2.7
10	8.5	8.3
Av.[b]	6.9	5.3

Table 2d. *Effect of type of anise oil on starter consumption with high citrus pulp and added* fat basal

Calf No.	Control	Imitation No. 1	Imitation No. 2	U.S.P.
	(av. lb. starter/calf/wk)[b]			
1	4.5	3.2	1.7	5.3
2	1.7	1.0	1.7	1.2
3	5.5	2.0	7.8	4.0
4	9.1	2.2	2.2	2.1
5	3.4	1.7	0.3	0.9
6	5.9	3.0	0.7	2.3
7	2.4	4.6	1.6	4.0
8	6.1	2.5	1.8	0.6
9	12.6	2.1	0.4	1.1
10	3.8	6.2	2.5	0.7
Av.[b]	5.8	3.6	2.9	2.6

[a] D.G. = degossypolized.
[b] Averages obtained from transformed data. The over-all averages were obtained from the average log values of all observations on each treatment.

There were several differences between the experiments, including the following: *(a)* A difference in time of several months existed. *(b)* In the first experiment, some of the starters contained much higher levels of imitation anise oil which could have had a tendency to more strongly repel the calves from all starters containing anise oil. *(c)* The basal starters were quite different. In the first experiment, 2% brown grease and a like amount of hydrogenated cottonseed

oil were used. In other similarly designed cafeteria experiments these fats have been shown to depress palatability drastically when large amounts are used *(3)*.

In the third experiment in which the basal starter contained no added fat, the calves ate an average of 6.9 lb/week of the control starter and only 5.3 lb. of the starter containing imitation anise oil No. 1. This difference was highly significant. Results of the third and fourth experiments are presented [Tables 2(c and d)].

The calves in the fourth experiment consumed the following pounds per calf per week of the four starters: *(a)* control, 5.8; *(b)* imitation anise oil No. 1, 3.6; *(c)* U.S.P. anise oil, 2.9, and *(d)* imitation anise oil No. 2, 2.6. The amount for the control was highly significantly higher than any other. Calves fed the starter containing imitation anise oil No. 1 ate highly significantly more than those fed starters with U.S.P. anise oil or imitation anise oil No. 2. The difference between the last two was not significant. Since the anise oil which found to be least detrimental was used in the other experiments, it is reasonable to suppose that either imitation anise oil No. 2, or the U.S.P. product which is used as a standard, would have been at least as detrimental had they been used.

The anise oils did not prove as detrimental in the fourth experiment—which also contained brown grease and hydrogenated cottonseed oil—as in the first experiment. Thus, while results of the third and fourth experiments can not be compared directly, it appears that the difference in the first two experiments can not be explained wholly as an interaction between the added fats and anise oil.

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SODIUM, POTASSIUM, CALCIUM, AND CHLORIDE DISTRIBUTION IN BOVINE SEMEN

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SUMMARY

Potassium is more concentrated within the sperm cells of bovine semen than in seminal plasma. Sodium and calcium are more concentrated in the seminal plasma than within the sperm cells. Varying concentrations of sodium and potassium in seminal plasma are characteristic of individual bulls and of the ejaculate, when consecutive individual ejaculates are considered. As an average, sodium and chloride concentrations are found to increase, and potassium and calcium concentrations to decrease, as increasing consecutive individual ejaculates are analyzed.

A number of workers have reported the concentrations of the bulk cations, sodium *(7–12, 15)* potassium *(7–12, 15)* and calcium *(6–12)* in semen *(6, 7, 9, 10)*, and seminal plasma *(8, 11, 12, 15)* of the bull *(8, 11, 12)*, ram *(11)*, boar *(9, 10)*, and human *(6, 7)*. A summary of the concentrations of these minerals is given (Table 1).

Although much work has been reported on these mineral concentrations, little consideration has been given to the differences in concentration of minerals in spermatozoa and in seminal plasma. Earlier published evidence from this laboratory indicates that the variations in amounts of these minerals in the fluids of the excurrent ducts of the male reproductive tract *(13)* may play a significant role in the metabolic behavior of spermatozoa *(4)* and in the long-

TABLE 1

Sodium, potassium, and calcium concentrations of semen and seminal plasma as reported by various workers

Workers	Substance analyzed	Na	K	Ca
		(av. mg/100 ml.)		
Nesmejanova *(10)*	Seminal plasma—bull	278	228	34
Rothschild and Barnes *(12)*	Seminal plasma—bull	258	172	37
Lutwak-Mann and Rowson *(8)*	Presperm fraction—bull	350	76	10
	Seminal plasma—bull	36–230	256	17–43
Nesmejanova *(10)*	Seminal plasma—ram	144	87	18
McKenzie *et al. (9)*	Whole plasma—boar	641[a]	243[a]	5[a]
Nesmejanova *(11)*	Seminal plasma—boar			
	1st fraction	312	54	6
	2nd fraction	234	100	9
	3rd fraction	305	56	7
Huggins and Johnson *(6)*	Semen—human	....	....	12–33
Huggins, Scott, and Heinen *(7)*	Semen—human	269	90	25

[a] mg/100 g. wet weight.

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time maintenance of their potential fertility while they are in the epididymis *(1, 5)*. Thus, evidence of changes in mineral composition of semen and the factors affecting these levels may lead to a better understanding of male reproductive physiology and of the items contributing to semen quality.

This investigation was conducted to determine the sodium, potassium, and calcium distribution in bovine semen and to determine some of the factors associated with changes in this distribution.

MATERIALS AND METHODS

Semen used in these studies was obtained by means of an artificial vagina from dairy bulls in the University of Illinois herd.

Sodium, potassium, and calcium determinations were made with a Coleman 21 flame phlotometer. This instrument was calibrated with five standard stock solutions for concentration ranges of sodium, potassium, and calcium corresponding to ranges which exist in reproductive tract fluids. The five concentrations of sodium ranged from 160 to 360 mg. per 100 ml. in increments of 50 mg. The five concentrations of potassium varied from 40 to 320 mg. per 100 ml. in increments of 70 mg., whereas those for calcium ranged from 10 to 50 mg. per ml. in 10-mg. increments. These five concentrations of the three minerals were made up in 15 combinations in order that resulting data might be analyzed according to the statistical design proposed by Box *(20.* The Box design permitted an accurate estimate of the variances associated with the linear and quadratic effects of the minerals involved on the photometer readings. This was accomplished by use of the model formula

$$Y_n = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3,$$

where Y_n = the transmission value for either Na, K, or Ca, the b's are the partial regression coefficients, and X_1, X_2, and X_3 represent the increment changes in mg. per 100 ml. of Na, K, and Ca, respectively.

Upon statistical analysis, the quadratic effects, i.e., X_1^2, X_2^2, X_3^2, X_1X_2, X_1X_3, and X_2X_3, were found to be relatively unimportant for the estimation of concentration of these minerals. Of these terms, the sum of the squares for sodium $\times$ sodium (X_1^2) in the sodium equation was the only one providing a significant ($P < .05$) deviation from linearity. However, this departure from linearity, if totally ignored, amounts to an extreme error of only about 5 mg. % at the extreme ranges of sodium values, either 160 or 360 mg/100 ml., and would be nonexistent at the mean value for sodium.

Therefore, the linear values, which were the only terms of importance, were placed in matrix form and the inverse matrix was calculated. To determine the mineral concentration in a sample, the light transmission values for sodium, potassium, and calcium obtained for the unknown were multiplied through by the inverse matrix. The values which resulted were the concentration in mg/100 ml. of the specific minerals.

The value of using this procedure lies in the fact that in each case a simultaneous solution is obtained for the concentration of sodium, potassium, and calcium. No standardizations of the concentration of one mineral prior to analysis of the next were necessary, for the effect of the concentration of one mineral on the transmission value obtained for another was accounted for algebraically.

Chloride, when determined, was analyzed by the silver-silver chloride electrode method of Chanin *(3)*.

Two studies were conducted. In the first, 16 first ejaculates of semen were collected at intervals approximately 1 wk. apart from four bulls (four ejaculates per bull). Immediately after collection, the semen was centrifuged for 1 hr. at approximately 700 g. A portion of the seminal plasma was drawn off and used for mineral determinations. The remainder of the plasma was drawn off and discarded. The packed cells were then drawn into a pipette by applying the laboratory vacuum to the pipette and the contents were subsequently discharged into a volumetric flask by applying a pressure system to the pipette. The cells adhering to the inner wall of the pipette were removed by repeatedly flushing the pipette with water distilled over glass which had previously been placed in the volumetric flask. Cell volumes were obtained by use of hematocrit tubes, which were also centrifuged for 1 hr.

In the second study, the semen and seminal plasma from four groups of ten individual consecutive ejaculates were analyzed for sodium, potassium, and calcium concentrations, and for chloride. After taking a sample of each of the 40 ejaculates for analysis, the semen was centrifuged and, subsequently, a sample of seminal plasma from each ejaculate was prepared for mineral analysis.

RESULTS

The concentrations of sodium, potassium, and calcium found in whole semen, spermatozoa, and seminal plasma in the first study are given (Table 2). Sodium and calcium were found in higher concentration in the plasma than in the spermatozoa, whereas potassium was found in higher concentration within the spermatozoa.

The differences between the concentrations found in cells and in the plasma were highly significant ($P < .01$) for each mineral.

TABLE 2

Concentrations of sodium, potassium, and calcium in whole semen, spermatozoa, and seminal plasma

(mg/100 ml.)

Bull	Na			K			Ca		
	Semen	Cells	Plasma	Semen	Cells	Plasma	Semen	Cells	Plasma
	(mg/100 ml.)								
A	300[a]	232	311	112	243	108	27	20	30
B	317	208	332	89	198	79	25	16	26
C	266	141	294	201	250	178	18	12	20
D	202	105	223	258	273	267	34	20	37
$\bar{x}$	271	173	290	163	241	161	26	17	28

[a] Average values of four ejaculates.

The average concentrations of spermatozoa (average of four ejaculates) in 1,000/mm^3 were 1,242, 1,752, 1,428, and 973 for Bulls A, B, C, and D, respectively. The average volumes of spermatozoa as determined by centrifugation in hematocrit tubes were 10, 12, 10, and 7%, respectively.

The average concentrations of sodium, potassium, and calcium in semen and seminal plasma of ten consecutive ejaculates of four other bulls are given (Table 3). The concentrations of chloride also are given for seminal plasma.

The correlations among sodium, potassium, and chloride concentrations in seminal plasma of each group of ten consecutive ejaculates are shown (Table 4).

TABLE 3

Average concentrations of sodium, potassium, calcium, and chloride[a] in the semen and seminal plasma of ten consecutive ejaculates

Ejaculate	Semen			Seminal plasma			
	Sodium	Potassium	Calcium	Sodium	Potassium	Calcium	Chloride
	(mg/100 ml.)						
1	286[b]	161	38	289	155	39	154
2	273	166	26	272	160	27	136
3	290	146	30	291	146	33	168
4	284	161	26	287	158	30	178
5	297	140	23	300	148	25	226
6	304	136	22	308	135	24	262
7	305	130	20	308	122	21	304
8	312	120	20	315	114	19	327
9	316	101	15	323	88	17	374
10	314	103	14	325	91	13	411

[a] For seminal plasma only.
[b] Average values of four ejaculates.

TABLE 4

Correlations among sodium, potassium, calcium, and chloride concentratioins in seminal plasma from groups of ten consecutive ejaculates

	Bull 1	Bull 2	Bull 3	Bull 4
Na–K	−0.92[b]	−0.88[b]	−0.40	−0.81[b]
Na–Ca	−0.51	−0.65[a]	0.60[a]	−0.68[a]
Na–Cl	0.97[b]	0.78[b]	0.04	0.90[b]
K–Ca	0.70[a]	0.17	−0.80[b]	0.93[b]
K–Cl	−0.98[b]	−0.78[b]	0.65[a]	−0.95[b]
Ca–Cl	−0.66[a]	−0.72[a]	−0.59	−0.89[b]

[a] $P < .05$.
[b] $P < .01$.

DISCUSSION

The average sodium, potassium, and calcium values reported in this paper (290, 161, and 28 mg. %, respectively) for seminal plasma of the bull are in close agreement with the values reported by other workers.

In view of the reports that potassium is found more concentrated in many types of cells than in their surrounding plasma, it is not surprising to find that this element is more highly concentrated within sperm cells than in seminal plasma; nor is it surprising to find sodium excluded from the cells to some extent *(16)*.

The significance of the concentration of potassium within the spermatozoa, and the exclusion of sodium from the spermatozoa in relation to storage qualities and fertilizing capacity, are not known. However, it is generally believed that both the concentration of potassium and the exclusion of sodium are the results of metabolic activity and utilization of substrate by the cell *(14)*.

The level of sodium and potassium in the seminal plasma of the ejaculate may also influence the concentration of these minerals in spermatozoa. When the potassium concentration in the plasma is about 270 mg. %, the concentration within the spermatozoa is only slightly greater (Table 2). However, when the plasma-potassium concentration is about 80 mg. %, the spermatozoa still maintain a concentration of about 200 mg. %. The concentration of potassium within spermatozoa fluctuates much less than the plasma-potassium concentration. Sodium concentrations within spermatozoa vary directly and as greatly as the corresponding plasma sodium values. Plasma contains approximately 120 mg. % more sodium than spermatozoa.

Two factors are evident in discussing the levels of sodium, potassium, calcium, and chloride in bull seminal plasma. These are the characteristics of the individual bull and, when consecutive individual ejaculates are collected, the ejaculate number. The accessory sexual glands of certain bulls accumulate potassium and calcium to a level much higher than that found in blood plasma. An increase in potassium and calcium is matched by a decrease in sodium to a level lower than found in blood plasma. It is suggested that energy is expended by the accessory glands to accumulate potassium and calcium in seminal fluids, and that sodium is displaced by these two minerals, assuming that a constant osmotic pressure is maintained.

As the ejaculate number increased in the series of ten, the average sodium and chloride concentrations in seminal plasma increased and the concentrations of potassium and calcium concentration decreased. However, consideration must be given to the fact that the changes which occur in concentration of sodium and potassium as ejaculate number is incresaed are largely conditioned by the level of these minerals as determined in the first ejaculate. If, in the first ejaculate, potassium concentration is approximately 90 mg. % and that of sodium approximately 320 mg. %, little change occurs in the concentration of these minerals in successive ejaculates. Although sodium and potassium concentrations may or may not change with increasing number of the ejaculate, chloride concentration increases in all cases. This phenomenon can result in a positive correlation of sodium and chloride in one case of a series of ten ejaculates, or in a positive correlation of potassium and chloride in another case. This is presented as evidence that the chloride concentration in seminal plasma of consecutive ejaculates may be largely independent of either the sodium or the potassium concentrations.

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DISTRIBUTION OF BULK AND TRACE MINERALS IN BULL REPRODUCTIVE TRACT FLUIDS AND SEMEN

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SUMMARY

Semen and seminal plasma and fluids from the testes, ampullae, and seminal vesicles have been analyzed for the concentrations of certain bulk and trace elements. Chloride concentrations also were determined for semen and seminal plasma of pooled ejaculates. The average values in milligrams per cent for semen and seminal plasma in the pooled samples of several consecutive ejaculates from individual bulls were, respectively: boron, 1.74, 1.73; phosphorus, 70, 40; magnesium, 9.8, 9.8; calcium, 22, 25; sodium, 227, 242; potassium, 173, 172, and chloride, 287, 330. The average of ten values in milligrams for semen and seminal plasma, respectively, of first ejaculates were: boron, 1.02, 1.48; phosphorus, 105, 55; magnesium, 12.1, 11.6; iron, .61, .35; calcium, 47, 51, and sodium, 263, 273.

Iron is mainly a constituent of spermatozoa throughout the bull reproductive tract and in whole semen. Phosphorus is found in concentrations of less than 10 mg. % in seminal vesicle fluids and in 35 to 45 mg. % higher concentration in seminal plasma. Zinc and manganese were found in concentrations of less than 0.2 and 0.04 mg. % respectively, in all samples analyzed.

Previous reports from this laboratory have shown the distribution of sodium, potassium, and calcium in male reproductive tract fluids *(14)* and of those elements and chloride in semen *(6)*. Earlier, Rothschild and Barnes *(13)* published on the levels and relationships of these same elements plus magnesium in seminal plasma.

A lower concentration of calcium and a higher concentration of potassium than in semen have been found in secretions from the tail of the epididymis *(14)*, where spermatozoa are known to have a long life span *(1, 8)*. Further knowledge of the mineral constituents of semen, seminal plasma, and reproductive tract fluids may lead to more effective means of controlling spermatozoan metabolism and storage qualities in vitro *(5)*.

The objective of this study was to determine the concentrations of boron, phosphorus, magnesium, iron, calcium, copper, sodium, potassium, zinc, and manganese in semen and seminal plasma and in testicular, ampullar, and vesicular fluids. Particular interest was centered on those ions not already examined in detail *(6, 14)*.

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MATERIALS AND METHODS

Source of materials. The semen used in these studies was obtained from dairy bulls by the use of the artificial vagina. Reproductive tract fluids were obtained from slaughter bulls, some of which were purebred dairy bulls from the University of Illinois herd, whereas others were of unknown breeding.

Methods of analysis. Mineral analysis was made with a Coleman 21 Flame Photometer and a Hilger large Liltrow Quartz Spectrograph. The methods used for the determination of sodium, potassium, and calcium with the flame photometer have been described *(6)*. A rotating disk method for solution analysis was used with the emission spectrograph.

Two methods were used to prepare samples for analysis by the emission spectrograph. The first method was hydrolysis by hydrochloric acid. In this method, the sample was added to an equal volume of concentrated hydrochloric acid and placed over a steam bath for 40 min. to hydrolyze the components of the sample. This method resulted in low values for copper and iron as compared with values obtained with dry ashing, the second method used. In this technique, the samples were dried and ashed according to the methods of Bills *(2)* and were then brought into solution for analysis.

With both methods, the solution for analysis was made up to a standard ratio of 1 ml. of sample (semen, seminal plasma, etc.), 0.5 ml. of a 1 *N* lithium chloride (internal standard), 1 ml. of concentrated hydrochloric acid, and 2.5 ml. of water. A minimum of 2.5 ml. of this solution was needed for one determination.

With this dilution technique, the spectral lines for potassium were not easily discerned; therefore, all potassium determinations were made with the flame photometer.

Chloride, when determined, was analyzed by the silver-silver chloride electrode method described by Chanin *(3)*.

In the initial phase of this study large samples of semen and seminal plasma were required for other purposes. Several consecutive ejaculates from individual bulls were pooled, with a collection period of 40 min. or less, and mineral determinations made on a sample from the pooled ejaculates. All of these composite samples were prepared for analysis by acid hydrolysis. Therefore, iron and copper values are not given. A portion of each composite sample was analyzed by the flame photometer, and chlorides were determined also. The plasma was separated from the cells by centrifugation.

In a second phase of the study, certain bulk and trace minerals were determined for testicular, ampullar, and seminal vesicular fluids and in semen and seminal plasma of single first ejaculates. These samples were prepared for analysis by ashing and all values reported in this phase of the work are from emission spectrograph. Consequently, no values for potassium were obtained.

RESULTS

The average values and standard deviations for concentrations of elements determined in the pooled semen and seminal plasma in the initial investigation are given (Table 1).

TABLE 1

Concentrations of some minerals and chloride in semen and seminal plasma[a]
(Pooled samples of several ejaculates from individual bulls)

	Semen		Seminal plasma	
	No. of samples	Average value	No. of samples	Average value
		(mg/100 ml.)		*(mg/100 ml.)*
B	8	1.74± 0.31[b]	8	1.73± 0.44
P	8	70.0 ±23.0	8	40.0 ±11.0
Mg	8	9.8 ± 1.9	8	9.8 ± 1.9
Ca	14	22.5 ± 6.6	14	24.6 ± 7.1
Na	14	227.4 ±58.0	14	242.0 ±53.0
K	14	173.0 ±91.0	14	172.0 ±92.0
Chloride	13	287.4 ±74.0	11	330.0 ±82.0

[a] Acid hydrolyzed.
[b] Standard deviation.

TABLE 2

Correlations between minerals for eight seminal plasma samples
(From part of data presented in Table 1)

Elements	P	Mg	Ca	Na	K	Cl
B	0.10	−0.01	−0.48	0.03	0.04	0.14
P		0.01	−0.02	−0.07	−0.03	0.00
Mg			0.78	−0.46	0.42	−0.57
Ca				−0.50	0.52	−0.67
Na					−0.94[a]	0.91[a]
K						−0.86[a]

[a] $P < .01$.

Complete data were available for eight samples of seminal plasma. Correlation coefficients were determined to express the interrelationships of these various elements. These are presented in Table 2.

The correlation coefficients for the mineral relationships generally were similar for whole semen. However, the concentrations of minerals in spermatozoa and in seminal plasma differ *(6)*. The concentration of spermatozoa in different semen samples may vary widely, so the relationships of minerals in whole semen may be less meaningful than the relationships of these same minerals in seminal plasma; thus, the coefficients for whole semen are not presented here.

Due to the limited size of samples obtained from the testis and ampulla, no attempt was made to separate the spermatozoa from the plasma. These samples were analyzed in toto. The seminal vesicular fluid does not contain spermatozoa but does constitute the source of a large portion of the seminal plasma.

The concentrations of the minerals found by emission spectrography in testicular, ampullar, and seminal vesicular fluids are presented (Table 3).

The data for the ten single ejaculates of semen and seminal plasma are presented in Table 4.

Since cellular volume in normal semen samples varies around 10%, the differences in mineral concentration between semen and seminal plasma must be increased by roughly ten times, to approximate the difference in concentration of a given mineral between the cells and the plasma.

The concentrations of zinc and manganese were less than 0.2 and 0.04 mg. %, respectively, in all samples analyzed.

TABLE 3

Mineral concentrations found in testicular, ampullar, and seminal vesicular fluids[a]

Elements	Testicular fluids		Ampullar fluids		Seminal vesicular fluids	
	No. of samples	Average value	No. of samples	Average value	No. of sample	Average value
		(mg/100 ml.)		*(mg/100 ml.)*		*(mg/100 ml.)*
B	12	0.80± 0.97[b]	3	0.59± 0.09	10	0.73± 0.16
P	12	229.00±23.00	3	328.00±38.00	10	10.0
Mg	12	14.9 ± 1.2	3	8.5 ± 1.2	10	17.5 ± 3.4
Fe	12	2.59± 0.74	3	1.08± 0.42	10	0.38± 0.28
Ca	12	4.6 ± 1.1	3	32.00± 7.00	10	70.00±13
Cu	12	1.36± 0.99	3	2.10± 1.50	10	0.95± 0.22
Na	12	178.00±24.00	3	137.00± 5.00	10	251.00±64.00

[a] Ashed.
[b] Standard deviation.

TABLE 4

Mineral concentrations found in semen and seminal plasma of ten first ejaculates[a]
(Two ejaculates/bull)

Elements	Semen		Seminal plasma	
	No. of samples	Average value	No. of sample	Average value
		(mg/100 ml.)		*(mg/100 ml.)*
B	10	1.02± 0.43	10	1.48± 0.77
P	10	105.00±40.00	10	55.00±18.00
Mg	10	12.0 ± 1.0	10	11.6 ± 1.0
Fe	10	0.61± 0.14	10	0.35± 0.29
Ca	10	47.00±14.00	10	51.00±15.00
Cu	10	0.60± 0.21	10	1.36± 0.49
Na	10	263.00±57.00	10	273.00±55.00

[a] Ashed.
[b] Standard deviation.

DISCUSSION

The values reported in this paper for phosphorus, magnesium, calcium, sodium, potassium, and chloride in seminal plasma of pooled ejaculates are in close agreement with those reported by Rothschild and Barnes *(13)*. The high negative correlation of sodium and potassium (−.94[3]) in seminal plasma of pooled ejaculates reported here corroborates the observations of Rothschild and Barnes *(13)* and our own earlier data *(4)*. However, the high correlations found for sodium and chloride (−.91[3]), and for potassium and chloride (−.86[3]), may be due to pooling of the ejaculates or to sampling. As has been shown in a previous report *(6)*, the relationships of sodium or potassium to chloride in single consecutive ejaculates fluctuate widely, not only from sample to sample within bulls but among bulls as well.

The concentration of phosphorus in the seminal plasma of either the pooled samples or in the single first ejaculates is approximately one-half the concentration found in whole semen. However, the concentration of phosphorus in seminal vesicular fluid is less than 10 mg. %. The difference between the level of phosphorus in the seminal vesicular fluid and seminal plasma is 30 to 45 mg. %. Thus, the other accessory glands must secrete this high phosphorus component. Lundquist *(9)* has reported a compound in bovine seminal plasma which liberates inorganic P and choline by enzyme activity on incubation. He speculated

[3] $P < .01$.

that this compound must originate from the seminal vesicles. Our data and the report by Mann *(12)* of a level of 9 mg. per 100 ml. of inorganic P in seminal plasma indicate that this probably is not the case.

The concentrations of iron reported (Tables 3 and 4) indicate conclusively that iron is much more concentrated in the spermatozoa than in the surrounding plasma. It is interesting to note that the average concentrations of iron in testicular fluids, ampullar fluids, and semen are, respectively, 2.59, 1.08, and 0.61 mg. %; whereas, the average concentrations in seminal vesicular fluids and seminal plasma are, respectively, 0.38 and 0.35 mg. %. The larger concentration of iron in spermatozoa may be largely tied up in the cytochromes *(11)*.

Zinc was not found in amounts of more than 0.2 mg. % in any of the samples analyzed, whether prepared by acid hydrolysis or by ashing. This is considerably less than the 2.84 mg. % reported by Dyrendahl *(7)* and lower than the 0.70 and 0.28 mg. % reported by Mann *(10)*, for ram spermatozoa and seminal plasma, respectively. Such results suggest that in bull semen zinc concentrations may fluctuate widely.

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VARIATIONS IN QUALITY OF LIQUID AND FROZEN SEMEN AT VARIOUS TEMPERATURES[1]

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SUMMARY

Ninety-two ejaculates of bovine semen were split into two portions; one portion was processed as liquid semen, the other as frozen semen. Each portion was further split into three parts which were incubated at 37, 20, and 7° C. The semen was evaluated microscopically for percentage of motile sperm and rate of progressive motility at specified intervals during a 24-hr. incubation period. Both frozen and liquid semen declined more rapidly in percentage of motile sperm and rate of motility at each higher temperature increment. At every incubation temperature, frozen semen showed a more rapid decline in both evaluation characteristics than did the corresponding liquid semen. At an incubation temperature of 37° C., all samples of frozen semen were immotile within 6 hr.

During the past few years, the characteristics of frozen semen have been the subject of considerable attention, and the short length of survival of frozen semen after thawing has been noticed by many people engaged in handling frozen semen. These observations have provided the reason for the recommendation that frozen semen be used as soon as possible after thawing.

There have been several experiments which have indicated that sperm longevity under incubation can be used to predict fertility. Ludwick *et al. (7)* found a correlation of 0.84 ± 0.03 between longevity and 60- to 90-day nonreturn rate when diluted semen was incubated at 100° F. Bratton *et al. (4)*, in comparing semen characteristics and fertility, obtained a significant correlation ($+0.207$) between livability at 5° C. until motility reached 30% and fertility at a 1:300 dilution but not at a 1:100 dilution. These correlations were obtained with liquid semen and do not indicate that the same might be true for frozen semen. O'Dell and Hurst *(9)* studied the decline in percentage of motile sperm in frozen and thawed semen during storage at 5° C. for five days. They concluded that thawed semen does not maintain good motility in storage and that the loss of motility is far more rapid than would be anticipated with samples of unfrozen semen.

This study was undertaken to provide information about the comparative longevity of frozen and liquid semen under incubation.

EXPERIMENTAL PROCEDURE

The semen used in this study was obtained from bulls at the Central Ohio Breeding Association, Columbus, Ohio. Ninety-two ejaculates from 38 bulls

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[1] The study was made possible through the assistance of Central Ohio Breeding Association, Richard Kellogg, Manager.

were used. All of the semen used in this study was collected by the bull stud for use as frozen semen.

The bull stud laboratory made the initial dilution of the semen for freezing with egg yolk–sodium citrate diluter. At this time, each semen sample was split; 3 or 4 ml. were removed and sent to the university laboratory, and the remaining portion was carried through the remainder of the freezing operation. At the university laboratory, the first portion (liquid semen) was held at 5–7° C. until approximately the time when the other portion was frozen. Then it was further diluted with egg yolk–sodium citrate diluter to the same extent as the frozen semen. Immediately afterwards, the sample was split into three equal portions for incubation at three different temperatures: 37, 25, and 7° C. Microscopic evaluations were made for percentage of motile sperm and rate of progressive motility at 0, 4, 8, and 24 hr. after the beginning of incubation. Two persons independently evaluated each sample and these evaluations were averaged.

The remainder of each original ejaculate was processed by the bull stud in accordance with their usual methods of freezing semen, which are as follows: The half-diluted semen was cooled to 4–6° C. during a period of 2–4 hr., then diluted to final volume by the slow addition of an equal amount of egg yolk–sodium citrate diluter containing 15% glycerol. The semen was allowed to equilibrate for 12–18 hr. before freezing. Freezing was accomplished by lowering the temperature from +5 to −10° C. in about 30 min., to −15° C. in 5 min. and, finally, to approximately −75° C. in about 5 min. After freezing, the samples were stored in a mechanical freezer at −83° C.

After storage for at least 2 wk., the frozen semen was thawed in cold water, incubated at the same temperatures as the unfrozen samples, and evaluated like the liquid semen. Microscopic evaluations were made at 0, 2, 4, 6, and 24 hr. after the beginning of incubation.

There was a total of 92 samples evaluated as both liquid and frozen semen at three different incubation temperatures.

RESULTS AND DISCUSSION

The mean percentages of motile sperm are presented (Table 1). The initial difference of 29.5% between liquid and frozen semen is somewhat higher than the

TABLE 1

Means of evaluations for per cent of motile sperm of 92 split ejaculates of liquid and frozen semen incubated at three temperatures

Time after beginning of incubation	Liquid semen			Frozen semen		
	37° C.	25° C.	7° C.	37° C.	25° C.	7° C.
(hr.)						
0	67.6	67.6	67.6	38.0	38.0	38.0
2	63.0[a]	65.3[a]	66.5[a]	17.3	18.1	30.0
4	57.8	63.1	65.0	5.0	10.8	27.0
6	52.6[a]	61.1[a]	63.5[a]	0	5.5	20.8
8	47.0	59.0	61.5	.....	.....	.....
24	0.5	44.0	57.0	0	0.4	10.8

[a] These figures were interpolated from the original data.

freezing losses observed in several other experiments *(1, 2, 3, 6, 8)*. This difference could be attributed to the fact that the semen samples used in this study were not selected from those used for breeding, but were used regardless of quality. Undoubtedly, some of them were not used for insemination because of poor quality after freezing.

As might be expected, there was a more rapid decline in quality at each higher incubation temperature for both liquid and frozen semen. Regardless of the initial difference, frozen semen declined in quality more rapidly than liquid semen at all temperatures. This difference was more pronounced at the higher incubation temperatures.

TABLE 2

Means of evaluations for rate of progressive motility of 92 split ejaculates of liquid and frozen semen incubated at three temperatures

Time after beginning of incubation	Liquid semen			Frozen semen		
	37° C.	25° C.	7° C.	37° C.	25° C.	7° C.
(hr.)						
0	8.49	8.49	8.49	7.12	7.12	7.12
2	7.69[a]	8.24[a]	8.37[a]	2.11	2.94	5.64
4	6.89	7.99	8.28	0.58	1.44	5.09
6	6.09[a]	7.80[a]	8.04[a]	0	0.67	4.04
8	5.28	7.61	7.82	.....	.....	.....
24	0.09	5.02	6.80	0	0.03	1.41

[a] These figures were interpolated from the original data.

In Table 2 are presented the means of evaluations for rate of progressive motility. These evaluations were made on a 0 to 10 basis, with 10 representing the highest motility (Table 2 indicates very much the same decline as Table 1.) The initial difference in motility between frozen and liquid semen is not as great as exhibited by percentage of motile sperm but, as before, frozen semen declined in quality more rapidly.

The rather low ratings of frozen semen incubated at 37° C., even after only a short period of incubation, can be attributed to the rapid and complete loss of motility in certain samples rather than to a generally diminished motility. At 4 hr. of incubation at 37° C., all of the liquid semen samples were still active, while many of the samples of frozen semen had become completely immotile. At 6 hr. of incubation, all of the frozen semen samples at 37° C. had become immotile.

At the present time, the cause of the rapid death of frozen semen during the incubation is a matter for speculation only. It might be assumed that such a short period of livability would not be conducive to good fertility, but this conclusion apparently is not true. In 1956, the last year in which simultaneous results with both frozen and liquid semen are available from Central Ohio Breeding Association, the 60- to 90-day nonreturn rate for frozen semen was 66.3%, compared with 66.6% for liquid semen. This represents approximately 93,000 first services to frozen semen and 78,000 to liquid semen.

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of both silages was higher in the 1957 crop than in the 1956 crop. Digestion trials in both years indicated that oat silage was a slightly lower TDN feed than corn silage, probably due to the higher crude fiber content.

In the first milk production trial (Table 2), little difference was found between the two silages with respect to butterfat test. The cows produced slightly more milk and gained more in body weight when fed the oat silage than when fed corn silage. These differences were statistically significant (1% level). This may have been due to the slightly higher rate of oat silage consumption.

TABLE 2

Influence of oat and corn silages fed to milking cows on average milk production, body weight, and feed consumption

	Trial 1		Trial 2	
	Corn	Oats	Corn	Oats
Milk produced *(lb/day/cow)*				
Preliminary period	29.3	29.1	35.2	35.4
Experimental period	29.9	32.5	28.6	27.9
Fat-corrected	28.0	30.4[a]	28.1	27.9
Butter test *(%)*	3.6	3.6	4.0	4.1
Last seven days	29.5	32.0	28.2	27.1
Fat-corrected	27.7	30.2[a]	28.4	26.7[a]
Body weight gain *(lb/cow/period)*	17.7	30.3[a]	27.1	0[a]
Feed consumed *(lb/cow/day)*				
Hay	9.8	9.3	7.3	7.2
Dry matter	8.5	8.0	6.3	6.2
Silage	55.0	44.0	83.1	69.0
Dry matter	14.7	15.5	20.8	18.3[a]
Gain	4.8	4.7	4.7	4.8
Dry matter	4.2	4.1	4.0	4.1
Total dry matter (DM)	27.4	27.6	31.1	28.6
Silage, DM/100 lb. wt.	1.30	1.44	1.60	1.46
Total roughage, DM/100 lb. body wt.	2.05	2.18	2.09	1.96

[a] Significant difference between rations at 1% level.

Data from the second milk production trial (Table 2) indicate that cows fed oat silage produced significantly less milk and gained significantly less in body weight than the cows fed corn silage. The average daily production of 4% FCM during the last seven days of each period for cows fed corn silage was 28.4 lb., as compared to 26.7 lb. for cows fed oat silage. These differences in milk production appear to be due to a lower consumption of oat silage dry matter. Cows fed oat silage consumed 18.3 lb. of silage dry matter per day as compared to 20.8 lb. for cows fed the corn silage.

There were some differences in the two milk production trials that probably influenced the results. The possibility exists that the oat silage used in the second trial was lower in milk-stimulating factors *(1)* than the oat silage used in the first trial. A higher rate of silage feeding was used in the second trial, but when the cows were fed oat silage they did not consume as much dry matter as when fed the corn silage. Thus, oat silage did not appear to be as appetizing as corn silage at high levels of consumption. All cows were offered the same amount of silage dry matter. A low rate of grain feeding was used in both trials and was essentially the same in the two trials.

Dairy heifers fed oat silage as the sole roughage made extremely poor rates of gain (Table 3), significantly poorer than beifers fed either alfalfa hay or corn silage. The consumption of roughage dry matter by heifers fed either alfalfa hay or corn silage was comparable, despite a lower dry matter intake by the heifers fed corn silage.

TABLE 3

Comparison of alfalfa hay, corn silage, and oat silage on the growth and feed consumption of dairy heifers (46-day trial)

Feed	Av. initial weight	Av. daily gain	Roughage consumption	
			Total	Dry matter
	(lb.)		*(lb/day)*	
Alfalfa hay	590	0.92	14.0	12.1
Corn silage	587	1.13	38.3	9.6
Oat silage	583	0.52	34.6	9.2
L.S.D.—5%	n.s.	0.26	.....	0.36

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TECHNICAL NOTES

COMPOSITION OF CASEIN IN THE SMALLEST CASEIN-CONTAINING PARTICLES OF MILK

In a previous publication *(1)*, analyses of centrifugally separated casein colloid fractions were presented which showed that over a broad size-range all of the particles contain a phosphoprotein which has the same phosphorus: nitrogen weight ratio, 0.0563. For the 12 samples analyzed, the standard deviation from this average is 0.00035. The smallest casein colloid particles, representing from 5 to 10% of the total casein, were not removed in the centrifuging times used; threfore, the results do not apply to these sizes. It has recently been reported that the constituents of the centrifugable casein micelles are in equilibrium with similar constituents free in solution *(5, 6)*. Therefore, it seemed worthwhile to make a careful analysis of the casein obtained from these smallest particles.

Portions of a sample of skimmilk were centrifuged in a Servall [1] angle-head centrifuge for 30 min., 60 min., and 2 hr., at an average centrifugal force of 27,000 times gravity, and in a 70-cc. capacity air-driven, air-floated bowl rotor for 90 min. at 490 r.p.s. In the latter case, the centrifugal force at the mid-point of the annulus was 39,500 times gravity, the time theoretically sufficient to remove all particles having sedimentation constants greater than 65×10^{-13}. The deposited colloids were discarded, the supernatant liquids from several runs accumulated, and aliquots analyzed. The caseins were then precipitated from the accumulated supernatants, at pH 4.6 in the usual way, redispersed in dilute alkali, reprecipitated, washed with water, dried and analyzed for total nitrogen, total phosphorus, and acid-soluble phosphorus.

Nitrogen was determined by a semimicro-Kjeldahl method *(1)*. Phosphorus was determined by King's semimicro colorimetric method *(2)*, using 1,2,4–aminonaphtholsulfonic acid after liquid digestion with concentrated sulfuric acid and hydrogen peroxide in the presence of selenium as a catalyst, the acid-soluble phosphorus on the hydrochloric acid extract. Separate test comparisons of the colorimetric method with a gravimetric, magnesium pyrophosphate method and a volumetric, ammonium phosphomolybdate method showed it to give results of equal accuracy.

The analyses of the four casein samples prepared as described, and other pertinent data, are given (Table 1).

It is clear that the O.P./T.N. ratio is the same for the noncentrifugable particles as for the others. The persistence of some acid-soluble phosphorus in the caseins suggests that these small particles are not free casein but, referring to the bowl rotor results, simply casein complex particles having sedimentation constants of the order of 65×10^{-13} or lower. This question could be settled by oxalate titrations of centrifuged serums by the Ling *(3)* or Pyne and Ryan *(4)* procedures.

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[1] The use of this trade name is for identification only and does not imply endorsement by the U. S. Department of Agriculture of the product or its manufacturer.

TABLE 1

Results of analyses of caseins prepared from centrifugally depleted portions of a skimmilk

Serum sample			Casein					
Centrifuging time *(min.)*	pH	Approx. % of total casein remaining	T.N.[a]	T.P.[a]	T.P./T.N.[a]	A.S.P.[a]	O.P.[a]	O.P./T.N.[a]
				Servall[b]				
30	6.72	60	15.08	0.916	0.0607	0.069	0.847	0.0562
60	6.60	40	15.23	0.962	0.0632	0.106	0.856	0.0562
120	6.62	25	14.96	0.880	0.0588	0.039	0.841	0.0562
				Bowl rotor				
90	6.72	8	14.08	0.817	0.0580	0.027	0.790	0.0561

[a] T.N. = total nitrogen; T.P. = total phosphorus; A.S.P. = acid-soluble phosphorus; O.P. = organic phosphorus.
[b] See footnote [1] in text.

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ADAPTABILITY OF THE FISHER-STERN ULTRACENTRIFUGE IN STUDYING THE SEDIMENTABILITY OF MILK PROTEINS

The Fisher-Stern Ultracentrifuge is a simple and relatively inexpensive centrifuge in which a plastic rotor spins in a vertical plane. The rotor is spun by a jet of air and the velocity is controlled by air pressure. The velocity is measured by an electronic tachometer with an accuracy of 1.5%.

Centrifugations were made in a room at 20–21° C. The thin cell was used for observation and pictures. The larger analytical cell was used to obtain samples of supernatant for analysis. Sedimentation times were taken as the time at which maximum depths of sediment were first attained as determined by measurement of pictures. The distance from the top of the cell to the top of the sediment represents the movement of the boundary. S values calculated by the application of the Svedberg equation, $S = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x}$, ranged from 430×10^{-13} to $57{,}000 \times 10^{-13}$. The value of 480 for a sample of untreated raw milk compares favorably with values 463 reported by Ford and Ramsdell *(1)* and 470 by Ford, Ramsdell and Landsman *(2)*.

As determined, these rates represent the sedimentation rates of the slowest fraction of the particles sedimentable at the velocities studied. Undiluted whole milk on which these determinations were made is relatively concentrated and complex, compared with solutions normally used to determine sedimentation constants in the measurement of molecular weights.

There was a great difference in the sedimentation patterns, depending on the type and treatment of the product. The amount of light transmitted through skimmilk at the start of the run was nearly the same as it was after maximum sedimentation. Further evidence for the role of fat in the amount of light transmitted through the cell was found in comparing raw whole milk with sterile homogenized milk. Fat from raw milk was thrown from suspension in the first minute of centrifugation, but in some cases a portion of homogenized fat remained in suspension after maximum sediment had been attained.

The pattern for sour coagulated milk appeared to be the expulsion of whey with no cream layer. Homogenized and sterilized 40% cream formed no sediment with all of the sedimentable solids in the cream layer. Fresh, sterile concentrated milk revealed a pattern similar to that of sterile whole milk. The rate became slower with age and increased viscosity. After a gel structure had formed during storage, the pattern appeared to be that of particles breaking away from a structure and settling quickly to the bottom of the cell. The fastest rate, $57{,}000 \times 10^{-13}$, was for a sterile concentrated milk (3:1) which had been diluted to the composition of the original milk.

Features of the Fisher-Stern Ultracentrifuge which makes it adaptable to the study of the sedimentation of milk proteins and the flotation of fat are: 1. The equipment is relatively inexpensive. 2. Maximum velocity can be reached in about 25 sec. 3. Sedimentation can be observed visually at the same time pictures are being taken. 4. Construction of the cell is such that convection currents do not interfere with sedimentation. 5. The plastic rotor and cell in a room at 20–21° C. do not increase in temperature due to friction with the air.

These studies are being continued to study the effect of processing on sedimentation in milk heated to different temperatures.

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THE HIGH-MELTING GLYCERIDE FRACTION FROM MILK FAT[1]

The metabolic fate of fats in the human diet is a current problem of considerable importance. One of the basic needs in this problem is for better analytical methods and more detailed information on the composition of both body and dietary lipids. Of the latter, milk fat is one of the most widely consumed and it is considered of particular interest that this fat consistently contains significant amounts of a high-melting glyceride fraction (HMFG) which has been only partially characterized. The discovery of this fraction and the information available on it stem from the work of Palmer and associates. A brief summary of its properties and distribution as reported by Jenness and Palmer *(4)* is as follows:

The HMGF is associated with the protein-phospholipid complex in the so-called membrane of milk fat globules. It occurs in milk fat to the extent of approximately 5% and in butter serum extracts, which represent a very small proportion of the total milk lipids, at a concentration of about 37%. HMGF preparations from buttermilk extract, butter serum extract, and butter fat were observed to have rather similar properies. Preparations twice crystallized from ethanol had iodine numbers of 5.0–7.1, saponification numbers of 198.8–204.0, and a melting range of 52–53° C.

Both the alcohol crystallization procedure *(4)* and crystallization from acetone were evaluated for preparing HMGF. The latter yields a very similar, if not identical, product to that obtained from alcohol, and enables use of smaller volumes of solvent with a given weight of fat. Data reported herein are based on the latter method, which involves crystallizing milk fat from five volumes of acetone at 2° C. and recrystallizing the solvent-free precipitate from ten volumes of acetone at 22° C. Studies at this laboratory have confirmed these properties of HMGF from milk fat produced at four different periods of the year, and have provided additional information:

As shown (Figure 1), infrared analysis indicated the HMGF to be a true triglyceride. Its spectrum (C) is virtually indistinguishable from spectra of tripalmitin (B) and tristearin (A). In order to further characterize the material, it was converted to its methyl esters by refluxing the acids, after saponification, with methanol using H_2SO_4 as catalyst. These esters (Sample A-1) were analyzed by gas chromatography, yielding the results shown (Figure 2). The peaks designated by Letters A through E correspond to the retention times, under the

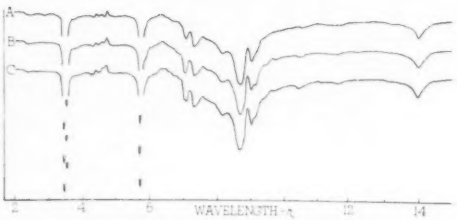

FIG. 1. Infrared spectra of tristearin (A), tripalmitin (B), and high-melting milk fat fraction (C). 5% (wt.) in CS_2.

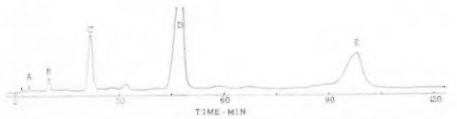

FIG. 2. Gas chromatogram of fatty acid methyl esters from the high-melting glyceride fraction. Peaks A to E correspond in retention times to the esters of capric, laurie, myristic, palmitic, and stearic acids, respectively. Operating conditions: sample—10 μl. column—8 ft. × ¼ in. packed with 80–110 mesh celite coated with 15% apiezon L, column temperature—230° C., helium flow rate—17 ml/min, chart speed—20 in/hr.

experimental conditions specified, for caprate, laurate, myristate, palmitate, and stearate, respectively. The inflection in the base line at retention time of 90 min. is in the area of oleic and linoleic acids. It is comparatively small, as would be expected from the iodine value (Hanus) of 2.0 for the original HMGF sample. At least six other minor peaks were evident. These may have arisen from esters of minor acids, a substantial number of which are known in milk fat *(6)*. They also may have arisen in part from decomposition products or unsaponifiable matter (for example, ketones; see below). Methyl esters (Sample B-1) from a second sample of HMGF were analyzed by gas chromatography on another instrument. Using the method of peak areas *(2)* from chromatograms, the approximate mole % composition of the methyl esters was calculated. These data (Table 1) indicate fairly close agreement between two samples analyzed separately in different instruments, and that saturated C_{16} and C_{18} acids account for over 75% of the acids in the HMGF. Acids of greater chain length than C_{18} were not present in detectable quantities. Hawke *(3)* has observed that methyl ester analyses by gas chromatography and by fractional distillation, a much more lengthy procedure, compare well.

[1] Authorized for publication on March 5, 1958 as Paper No. 2240 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

TABLE 1

Composition of the fatty acid methyl esters from two samples of high-melting glyceride fraction based on peak areas from gas chromatograms

Acid chain length	Mole %	
	A–1	B–1
C_{10}	0.3	0.7
C_{12}	1.4	1.6
C_{14}	12.1	13.6
C_{16}	50.5	51.6
C_{18} saturated	25.5	30.4[a]
C_{18} unsaturated	4.1	
Unassigned peak areas	5.9	1.1

[a] Includes C_{18} unsaturated acids.

HMGF was analyzed for polyunsaturated acids by the method of Brice *et al. (1)*, which involves ultraviolet spectral analysis of the fat before and after alkali isomerization. These data (Table 2) show very limited quantities of such acids to be present and suggest that the small iodine number of the fraction is contributed mainly by monolinoic acid.

TABLE 2

Polyunsaturated acids in a sample of high-melting glyceride fraction from milk fat as analyzed by the method of Brice et al. (1)

Type of acid	Wt. %
Linoleic	0.005
Linolenic	0.032
Arachidonic	0.022
Conjugated diene	0.069
Conjugated triene	0.004
Conjugated tetraene	0.000
Total	0.132

The amount and nature of the unsaponifiable matter from the HMGF was considered worthy of investigation. Unsaponifiable matter averaging 0.4% was observed. Infrared spectral analysis of the unsaponifiable in CCl_4 revealed distinctive absorption at 1,710, 1,258, 1,093, and 1,012 $cm.^{-1}$. On the assumption that the absorption at 1,710 $cm.^{-1}$ arose from ketone carbonyl, unsaponifiable was reacted with 2,4–dinitrophenyl (DNP) hydrazine reagent. The reaction product was paper-chromatographed by the method of Klein and de Jong *(5)*. Analysis of the unsaponifiable from four different samples of HMGF by this technique revealed in each case a derivative moving in corresondence to that of pentadecanone–2. A more faint spot corresponding to that of tridecanone–2 also was noted. These two ketones were identified, as their DNP–hydrazones, in the unsaponifiable matter of milk fat by their spectral properties, behavior on paper chromatograms, melting points, and mixed melting points with authentic derivatives. Details of this latter work will be published subsequently.

We are very grateful to S.R. Lipsky, Yale Medical School, New Haven, for gas–chromatographic analysis of Sample A-1 (Figure 2 and Table 1), and to K. P. Dimick, Wilkens Instrument and Research Inc., Berkely 4, Calif., for analysis of Sample B-1 (Table 1).

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EFFECT OF POTASSIUM OROTATE AND METHIONINE ALONE AND WITH VARIOUS ANTIBACTERIAL AGENTS IN A HIGH COLOSTRUM DIET FOR YOUNG CALVES[1]

[1] Florida Agricultural Experiment Station Journal Series No. 652.

Supplementary potassium orotate with methionine appeared to stimulate growth of yearling heifers *(9)* and young calves *(10)*. Orotic acid per se also was effective as a growth promotant when fed with methionine to dairy calves *(7)*. Orotic acid is a growth factor for some bacteria *(1, 5)*. It is possible, therefore, that its effect on animal growth resulted indirectly from stimulation of symbiotic organisms. Dietary antibiotics *(2)* and other antobacterial agents *(3, 8)* also have increased growth rate in dairy calves, possible by inhibition of antagonistic microorganisms. Hence, an increase in the growth rate of calves may have resulted from stimulation of some organisms and depression of others. This experiment was designed to obtain information on the effect of feeding supplementary potassium orotate (a bacterial growth factor) simultaneously with various inhibitors of bacterial development under especially favorable environmental conditions.

Thirty-six new-born Guernsey and Jersey calves were allotted to six groups which were identical as to sex and breed and similar in weight and height at withers. The calves were managed and fed as in previous work *(11)*. The system included confinement in individual pens and feeding of pure colostrum through four days of age, one-half colostrum and one-half skimmilk through 21 days, and skimmilk until calves were 60 days old. All milks were fed at the rate of 9% of body weight, two equal portions daily. Chopped alyce clover hay and mixed concentrates were offered free choice. Two hundred and twenty milligrams each of methionine and potassium orotate per hundred pounds body weight were fed to all subjects other than the controls. Forty-five milligrams of chlortetracycline or 90 mg. per hundred pounds live weight of one of the other antibacterial agents were dispersed in the milk fed to calves in respective groups, as follows: Group 3, chlortetracycline; Group 4, *para*-amino salicylic acid (PAS); Group 5, isoniazid, and Group 6, erythromycin.

Daily records included observations on general health and consumption of feed. Body weights and heights at withers were recorded on the fourth day postpartum, and at weekly intervals thereafter. All data were analyzed statistically by analysis of variance *(6)*.

No scouring was observed. All calves were sleek and alert, and no differences in appearance between groups were discernible. Average gains in body weight were higher in all treated calves than in the controls (Table 1). However, these differences were not statistically significant. Changes in height at withers and efficiency of feed utilization appeared to be unaffected by any of the supplements.

That general physical stress was at a minimum is indicated by the exceptionally good growth of the controls, which gained an average of 131% of weight considered as normal (Ragsdale Standard, *1*). Supplementary growth factors seldom have resulted in performance superior to that exhibited by the control animals in this work. Percentagewise, the present differences seem to be of some importance. A larger number of experimental subjects would make the data more convincing. It seems likely, however, that the need for supplementary

TABLE 1

Relation of potassium orotate fed alone and with antibacterial agents to growth and feed consumption by calves

Ration	Average total feed consumption				Body weight			Height at withers		
	Diluted colostrum	Skimmilk	Hay	Concentrates	Initial	Gain	Range in gain	Initial	Gain	Range of gain
	(lb.)				(lb.)			(cm.)		
(1) Control	92.8	289.3	8.4	52.5	60	51	46–78	69	9	7–12
(2) Potassium orotate + methionine	97.4	321.7	9.0	62.8	60	59	51–77	70	9	8–11
(3) (2) + chlortetracycline	99.6	318.9	11.3	58.9	65	58	42–76	72	9	7–11
(4) (2) + PAS	98.3	327.3	7.4	69.6	65	57	51–69	71	9	7–10
(5) (2) + isoniazid	94.0	302.8	14.0	61.0	58	57	41–72	68	10	8–10
(6) (2) + erythromycin	95.3	304.3	11.5	61.0	59	56	46–63	69	9	8–11

growth factors was at least partially eliminated by the colostrum.

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FERTILITY OF BOVINE SEMEN EXTENDED AT 28–30° C. IN EGG YOLK–CITRATE WITH OR WITHOUT GLYCERIN[1]

Several workers *(1, 2, 3)* have reported that glycerin added at 5° C. to partially extended semen prolongs the livability and fertility of the spermatozoa when the semen is stored at 5° C. To be practicable at this bull stud, this addition at 5° C. of glycerin to semen which is to be stored and used as a liquid, probably would have to be performed in a refrigerated room, a costly item. The experiments to be noted here were conducted to determine whether glycerination of semen at 28–30° C. would be effective in improving livability and fertility of spermatozoa.

At this laboratory, in April and May of 1957, semen livability trials were conducted to determine simultaneously not only the effects of glycerination at different temperatures but also the effects of two variations in the rate at which glycerin may be added when extending semen to a constant motile sperm count and glycerin content. Semen samples were divided into two equal portions. In a single operation, one portion was completely extended at 28–30° C. in glycerinated yolk-citrate (1:3 yolk-citrate plus 8% glycerine by volume). The other portion was partially extended at 28–30° C. in yolk-citrate and 3 hr. later completely extended at 5° C. with an equal volume of a 16% glycerin-citrate solution, added in three equal portions at ½-hr. intervals. Motility estimations were made daily through the 15th day of storage. After seven days of storage the mean motility for 34 semen samples was 38% for the samples completely extended at 28–30° C., and 37½ for those partially extended at this temperature and later completely extended at 5° C.

Therefore, from September 15, 1957, through November, 1957, a field trial was conducted to compare the fertility of semen glycerinated at 28–30° C. with that of non-glycerinated semen. A split-ejaculate, county association–reversal technique was used. Freshly collected semen was added to both glycerinated and non-glycerinated yolk-citrate at 28–30° C.; the semen was then cooled slowly to 5° C. From September 15 through October 31, 35 associations received glycerinated semen, and 37, non-glycerinated semen. During November, the two groups of associations were reversed. The results of the field trial are presented (Table 1).

TABLE 1

Fertility of bovine semen extended at 28–30° C. in glycerinated or nonglycerinated egg yolk–citrate

Extender	First services	60–90 Day non-return rates
	(No.)	*(%)*
Glycerinated	5,612	69.9
Nonglycerinated	6,044	68.3

[1] Contribution No. 264, Kansas Agricultural Experiment Station, Manhattan.

The fertility of bovine semen extended in glycerinated yolk-citrate at 28–30° C. was slightly higher than that of semen extended in yolk-citrate at the same temperatures. The difference of 1.6 percentage units in the 60- to 90-day nonreturn rates was not statistically significant, but approached significance at the 5% level ($X^2 = 3.45$, 0.10 P 0.05).

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OUR INDUSTRY TODAY

A FULLY MECHANIZED METHOD FOR THE MANUFACTURE OF CHEDDAR CHEESE[1]

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Interest in the mechanization of cheese-making has led to the Steinecker vat method *(15)* for soft ripened cheese, the Stine process *(8, 9)* for Domestic Swiss, and the DeLaval centrifuge method *(11)* for soft unripened cheese. Despite these advances, most present-day cheese-making, particularly Cheddar and cottage, is not highly mechanized.

The use by Cornell researchers *(1, 2)* of salt-tolerant enterococci as cheese starters, along with the obvious advantages of these bacteria to continuous practices, stimulated further interest in the development of mechanization of Cheddar cheese-making. Walter *et al. (13, 14)* reported on such a method, along with later modifications having as key features a supplementary enterococci starter and the pressing of hooped, salted curds under warm whey. Australian workers *(4)* readapted this USDA method, but retained many of its key features. In the latest experimental attempt to produce a labor-saving Cheddar cheese method, the United States Department of Agriculture *(12)* has recently suggested reverting to the traditional manufacturing procedure, but with the exclusion of hand-cheddaring. Instead, the curds would be overlaid with warm whey, followed by pressing with metal plates and weights for a period of 2 hr. while the whey was draining off.

Scandinavians, for many years, have pressed curds under warm whey for a ripened hard cheese, and such a practice is common with certain cheese in Central American countries. One of the two Stine patterns in 1950 (9) for Domestic Swiss was concerned with the pressing of curds into one large block under warm whey, and a similar type of patent *(10)*, issued to the same inventor, exists for Cheddar cheese. Apparently, the latter never has been translated into general industrial practice.

Recently, Czulak and Hammond *(3)* designed an experimental apparatus to fuse curd blocks mechanically in the absence of excess whey, a step in a cheese-making method not yet completed. In England, the Desco method for Cheddar cheese *(7)* is well-mechanized, especially at the point of salting, but the cheddaring step is still carried out by hand.

This report presents a detailed account of the principles and performance of a commercially tested, mechanized method for Cheddar cheese manufacture called the Ched-O-Matic method.[3] The method has been in commercial operation since 1956 and was tested on a large scale for several years earlier.

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[1] With the technical assistance of Frank V. Kosikowski, Department of Dairy Industry, Cornell University, Ithaca, N. Y.

[2] Senior author—deceased February, 1958.

[3] Patents pending.

RESULTS

The Method

Principles. An integrated, mechanical movement of the curd components of cheese produced with lactic starter through draining, cheddaring, salting, and hooping characterizes the present method. Hydraulic compression of drained curds into one large block and held thus, but not under whey, while acidity develops, replaces the laborious, manual maneuvering of many small curd blocks associated with traditional cheddaring. Reduction in size of the single large block is made in the press by an attached vertical and horizonal knife actuating mechanism. The resulting smaller curd slabs fall on conveyors and are shredded, washed, and salted automatically while moving to the hooping area.

Procedure. The Ched-O-Matic process for Cheddar cheese has been conceived as a unit operation. A full unit requires, in addition to conventional cheese vats, one rotary drainage tumbler drum, two mechanical hydraulic whey extrusion presses, and accompanying shredder and salt sprayers.

With the present commercial process, a half-unit was employed consisting of one rotary tumbler drum and one press. This half-unit forms a single curd block of approximately 1,200 lb. from one vat of 13,000 lb. of milk. Steps required, based on this capacity, are expressed in the following paragraphs and in Table 1.

Setting of milk, cutting, and cooking curd. Raw, heat-treated milk of about 3.5% fat was used for most runs, but the method has also been designed for the use of pasteurized milk.

A vat of milk, 13,000 lb., is set at 88° F.,

TABLE 1

Typical time table of Cheddar cheese manufacture for three vats

Elements of make-up	Cheese manufactured on April 28	Cheese manufactured on April 25	Cheese manufactured on April 20
Setting of milk			
Lb. of milk	13,000	13,000	13,000
Per cent lactic starter	1.8	1.8	2.1
Oz. Rennet	30	30	30
Titratable-acidity	0.18	0.18	0.18
Marshall cup	2.5	2.5	2.5
Cutting and cooking	4 hr.	3 hr. 25 min.	3 hr. 45 min.
Size of cut *(in.)*	¼	¼	¼
Time of cooking *(min.)*	30	30	30
Peak temperature	101	101	101
Final stirring-out time *(min.)*	5	10	10
Per cent whey acidity at pumping	0.14	0.14	0.14
Block pressing time	2 hr.	1.5 hr.	1.75 hr.
Milling and salting per cent acidity	0.59	0.54	0.60
End of hooping	< 20 min.	< 20 min.	< 20 min.

using about 2% active lactic acid starter and enough rennet to form a solid curd in 20 min.

The firm curd is cut with ¼-in. wire knives and the curds cooked to about 100° F. over a 30-min. period. Curds are stirred out in the vat for 5–10 min. at the end of cooking.

Draining. The mixture is then pumped in its entirety through sanitary pipe lines into the open end of a large, horizontal, stainless steel, finely perforated drum, pitched slightly forward (Figure 1). As the drum revolves, the tumbling action of the curds frees the whey,

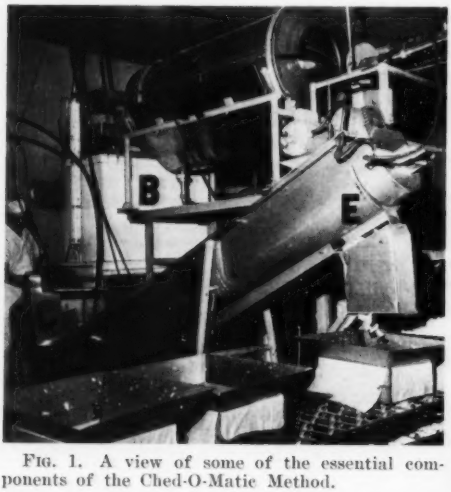

FIG. 1. A view of some of the essential components of the Ched-O-Matic Method.

A—Rotary Tumbler Drum
B—Hydraulic Press
C—Milling Machine
D—Screw Conveyor with Salting Jets
E—Hoop Filler

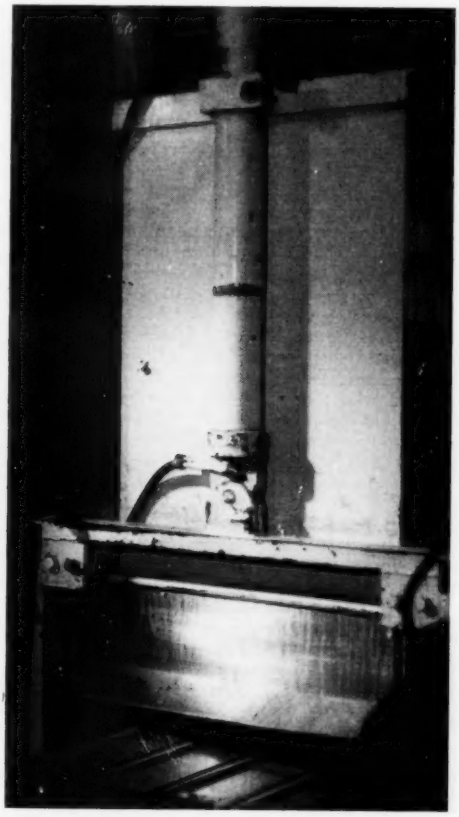

FIG. 2. End view of hydraulic press. Vertical knife blade is at bottom.

which escapes through extremely small pores into storage tanks, leaving behind the curd particles. The forward end of the drum is then opened to discard the mass of curds into the mechanical, hydraulic press equipped with a system of top and bottom screens (Figure 2). The curd rests in this press without surrounding whey.

Formation of cheese texture in hydraulic, whey extrusion press. Pressure is applied mechanically to the mass of curd in the press at a rate of 0.45 to 1.75 p.s.i.g. The general objective here is to attain within the press a final whey acidity of 0.5–0.65%, and a moisture concentration of 35–40% in about 100 min.

While in the press the large curd block is never turned. The extruded, unsalted whey passes through perforations to separators and storage tanks.

Attainment of optimum acidity and moisture in the massive, stationary curd block results in a texture and stretch usually associated with good blocks of curd obtained by the traditional cheddaring step.

A unique movable parting knife and horizontal partition are located at the bottom of the press. As soon as the curd block is properly formed and textured, determined by acidity measurements, the parting knife cuts across, actuated by a hydraulic cylinder, and the cut lower section of curd is pushed outward by an extrusion piston. As the curd section leaves a cut-out section of a side wall, a vertical knife is moved upward by the action of hydraulic cylinders so that small blocks of curd are cut off directly onto a moving conveyor. The block size at this point can be varied, but nominally 24 by 6 by 2-in. blocks were found most suitable. These automatic cutting movements, horizontal and vertical, quickly consume the entire 1,300-lb. curd block.

Milling. The blocks are conveyed directly into a milling machine which deposits the shredded particles into an elevated Archimedean screw conveyor for salting (Figure 3).

Salting. The inner housing of this screw conveyor is fitted with a series of jet sprays. These jets spray clean, warm water or saturated salt solution in any combination at pressures insufficient to shatter curds. As the milled curds are propelled upwards, the jets open up; the first series may wash the curds with warm water, while a second series infuses the cheese with brine (Figure 4). Temperatures of sprays nominally are 100 to 104° F., but higher or lower temperatures may be used. All brine jets need not operate simultaneously and if increased salting is required, additional jets may be brought into play. Surplus brine is drained off through a groove on the underside of the screw conveyor and need not be combined with the regular whey supply.

Pressing and ripening. Salted curds move rapidly to the top of the screw conveyor and continuously drop through a hopper into round or square hoops of any size desired (Figure 1, E). This method of hooping is especially suited for small molds. The properly hooped cheeses are pressed conventionally and ripened as for cheese made in the traditional manner.

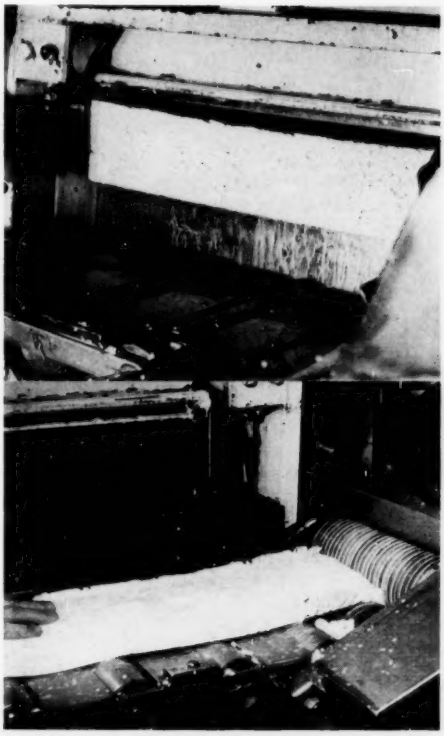

FIG. 3. A. Block of curd being extracted and ready for vertical cutting. B. Cut slab entering milling machine.

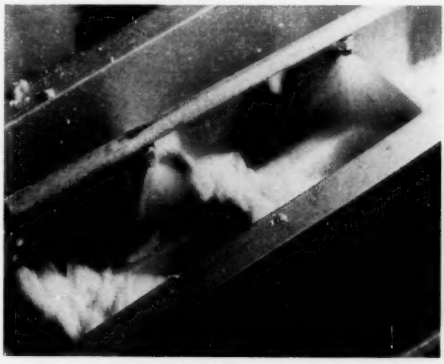

FIG. 4. Water and salt spray in screw-conveyor housing.

Quality, composition, and yield. Since its inception, approximately 600,000 lb. of regular and washed-curd Cheddar cheese have been manufacured with the Ched-O-Matic method. These cheese have been purchased by buyers—not connected with the production firm—of large amounts of cheese at regular market prices, and have been distributed through normal consumer channels in the United States.

The quality of the cheese made in this fashion has been uniformly good-to-excellent (Table 2). The flavor was true to type and generally without any obvious defects. A slight whey taint, evident in a small percentage of earlier cheese, was eliminated in later cheese by the more effective utilization of wash water and brine sprays.

The body of the cheese was firm, and during ripening it developed a waxy texture. A few mechanical holes of small size were evident throughout the cheese (Figure 5). This characteristic is not entirely undesirable, in light of the fact that experimental Cheddar cheese produced by the USDA mechanized method *(13)* was criticized because its compactness and closeness were too closely identified with that of processed cheese.

Control of composition to fit into existing standards of identity and normal custom was achieved without difficulty. The salt and moisture concentrations of successive cheese produced during a given period (Table 2) are similar to those usually obtained in traditional cheese-making.

In northern New York State, where these cheeses were manufactured, an earlier survey of 15 factories *(6)* listed the average yield of Cheddar cheese from 3.5% milk as 8.92%. The new method of cheese-making maintains and,

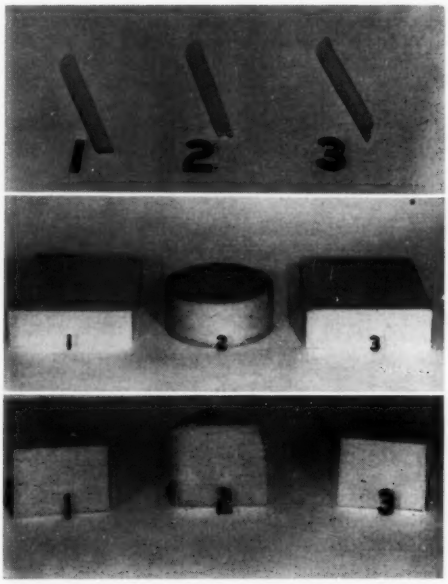

FIG. 5. Cheddar cheese, 1 week old, by Ched-O-Matic method.

1—20-lb. rindless by Chedo-O-Matic. 2—12½-lb. wheel by Ched-O-Matic. 3—20-lb. rindless by traditional process. All cheese have compact bodies with a few mechanical openings.

TABLE 2

Quality and composition of fresh cheese made by Ched-O-Matic[a]

Cheese manufactured on	Quality by commercial grader	Moisture	Salt
	*(grade)**	*(%)*	
April 28			
Vat—1	No. 1—Slightly open	37.4	1.6
Vat—8	No. 1—Slightly open	35.6	1.5
April 27			
Vat—1	No. 1—Open	37.6	1.4
Vat—8	No. 1—Slightly open	38.0	1.2
April 26			
Vat—1	No. 1—Open	38.0	1.2
Vat—8	No. 1—Open	37.6	1.4
April 25			
Vat—1	No. 1—Open	37.6	1.2
Vat—8	No. 1—Slightly open	38.0	1.3
April 22			
Vat—6	No. 1—Close	37.4	1.3
April 21			
Vat 1	No. 1—Firm, slightly open	37.8	1.6
Vat 7	No. 1—Firm, slightly open	37.6	1.4
April 20			
Vat—1	No. 1—Firm, slightly open	36.8	1.4
Vat—8	No. 1—Open	37.6	1.5

* No. 1 Grade = AA on U.S. Government Standards, or top-quality cheese.

[a] Not more than two commercial vats were being run daily by Ched-O-Matic on this test trial. Above table includes all cheese made during week April 20–28, 1958.

in most instances, exceeds this average yield (Table 3).

TABLE 3

Yield of Cheddar cheese made by Ched-O-Matic

Date of make[a]	Yield[b]
	(lbs. cheese/100 lb. milk)
Period of April 5–12, 1958	
April 5	9.0
April 6	9.0
April 7	8.9
April 8	9.1
April 9	9.1
April 10	9.0
April 12	9.2
Period of May 26–June 5, 1958	
May 26	9.4
May 27	9.3
May 28	9.5
May 29	9.6
May 30	9.5
May 31	9.2
June 1	9.3
June 2	9.3
June 3	8.9
June 4	8.9
June 5	9.2
Average for combined periods	9.2

[a] Represents all cheese made by Ched-O-Matic during two periods. This includes 46,700 lb. Cheddar cheese made in 33 vats, as blocks, flats, and exports.

[b] All yield values calculated to 3.5% fat milk and 37.0% moisture in cheese.

Economic aspects. Certain economic advantages are apparent in the use of the Ched-O-Matic method. From setting of milk to termination of pressing, an average period of 3 hr. and 51 min. was required. This represents at this point no drastic savings of time, but a pronounced reduction in labor both in number of personnel and in movements. From the pressing of the curd block through salting and final hooping, less labor is required and a saving of about 40 min. per vat is effected because of the mechanical flow of the curds. The over-all saving in time over traditional methods is about 1 hr.

Under optimum conditions three complete units of the size described here—with appropriate vats—conceivably could, with two shifts of workers, turn 250,000 lb. of milk into Cheddar cheese daily. Accounting analyses of the present one-half-unit operation have indicated a reduction in costs of about one cent per pound of cheese.

An additional feature of the present method is the much greater control attained by one operator over the product quality and sanitation.

DISCUSSION

Perhaps no other need in the cheese industry has become so paramount as that of designing automatic equipment which will transform milk into good quality cheese, true to type, without supplementary manual manipulations and at reasonable costs. This has been clearly stated by Keestra *(5)* and it was the guiding motivation of the present research project.

The current recommendations regarding cutting and cooking, the acid development rate, and the degree of salting are, of necessity, based on characteristics displayed by New York State Cheddar cheese. Other types of hard cheese, or Cheddar cheese from other regions, might react slightly differently to mechanical handling, but with the demonstrated flexibility of the Ched-O-Matic unit no great problems are anticipated.

A characteristic feature of the Ched-O-Matic unit is the whey expulsion press, with its unique system of automatic cutting knives and its movable lower partition. Recently, Czulak *et al. (3)* reported on an experimental curd fusion apparatus for Cheddar cheese which forms and discharges a block of curd in 15 min. This is in contrast to the 100 min. or more recommended to hold a block in the Ched-O-Matic press. For their initial work, the Australian investigators *(3)* have chosen to use a supplementary enterococci starter, *Streptococcus durans*, which grows well in high salt concentration. In 1948, Dahlberg and Kosikowski *(1)* first showed that enterococci had useful functions in cheese-making. Working with *Streptococcus faecalis*, a bacteria almost identical to *S. durans*, they state *(2)*: "The usual commercial lactic starter may or may not develop sufficient acid in the presence of salt. The ability of *S. faecalis* to develop acid rapidly after salting curd is very important in several regards. It permits the addition of salt to cheese curd containing insufficient acid, with assurance that the correct acidity will develop in the cheese during (final) pressing. . . ."

Thus, in the present instance, it is merely a choice of certain fundamental cheese-making principles, i. e., *(a)* to develop acid in the whey extrusion press and salt a fully textured curd, or *(b)* to use enterococci starter, press the block without much acid development, salt the curds, and look forward to proper acid and body development in the final hoop pressing. Because most New York State cheese is from raw, heat-treated milk which requires no enterococci starter, the choice was that of the first, and the one upon which most of the present practical experience is based. Under other circumstances a 10- or 15-min. pressing period, too, could be fitted into the Ched-O-Matic production scheme.

Another problem, perhaps more formidable than pressing, dealt with continuous salting. Dry salt applications as practiced with the Desco apparatus *(7)*, though not impossible, are not the most suitable for the present new method. The large block of curd entraps some whey which is not entirely removed following

milling. Dry salting alone would result in cheese with whey taint. After extensive trials, it was discovered that a system of nozzle jets spraying warm water and brine removes this small amount of whey effectively while injecting the required amount of salt in the cheese. Also, as the temperatures of these water and salt sprays can be varied between 90 and 115° F. or higher, a final opportunity for moisture and temperature control of the curds is possible. Dry salting with electronic salting devices is by no means impractical if accompanied by prior clear, warm-water jet-spraying of curds to remove whey.

Complete salt absorption into the moving cheese curd is marked by the speed of its achievement. A ½-min. exposure of the moving curds to a series of four sprays of saturated salt solution will produce cheese of 1.5% salt concentration. The success of this application is undoubtedly linked to the initial porous, almost spongy, nature of the curd slabs displayed upon extrusion from the whey expulsion press (Figure 3, A). An interesting phenomenon observed with the Ched-O-Matic method is the striking conversion of these small slabs of porous curd to close, well-knit bodies during hoop pressing and ripening. This abrupt reversal in physical quality may contain the key to the little-known factors that control the mechanics of curd body behavior of cheese.

Brine salting, as practiced in the Ched-O-Matic method, avoids a basic criticism leveled earlier at the USDA method *(13)* for Cheddar cheese manufacture. No large quantity of heavily salted whey is accumulated and the surplus brine solution need not be diverted into fresh whey channels.

In the present state of development of the new method, a heavier than usual loss of salt results. These losses, however, do not raise the cost of cheese making exorbitantly, nor need they be sustained in future operations. For example, 250 lb. or more of cheese salt may be required to make sufficient brine to spray the cheese from one full unit, or 2,600 lb. This cheese salted to about 1.5% retains only 42 lb. of salt, whereas 208 lb. are not used. In contrast, the traditional practice for the same cheese poundage requires 84 lb., of which about 40 lb. are removed with the whey. At the present price of salt, this would amount to 0.12¢ additional per pound of cheese with the Ched-O-Matic process, a cost which, in turn, is more than offset by savings in labor and time. Furthermore, in large operations, daily regeneration of these brine solutions by passage through separators, reinforcement with additional salt, and then recirculating over a second unit of cheese is possible.

SUMMARY

A fully mechanized new method for the manufacture of Cheddar cheese, based on rapid drainage of curds in a special rotary drum, formation of large curd block, pressed hydraulically in the absence of surface whey, and automatic milling, salting, and hooping, is presented. This method, called Ched-O-Matic, has undergone extensive and large scale research investigation. The finished cheese has become fully acceptable to the trade. A matured cheese has a flavor typical of good Cheddar and displays a firm, waxy body and texture showing a few mechanical holes. The latter characteristic, if not excessive, is a desirable trait of natural Cheddar cheese.

Advantages in the method lie in its easy line-flow principles of materials passing through automatic machinery. Total personnel can be reduced and peak intensity of remaining labor can be coordinated so that handling of materials is less laborious. Cheese-making is shortened by almost 1 hr., saved mainly during salting and hooping, and a more favorable environment is created for the cutting of unit costs. In addition, easier and more effective control over product quality and sanitation are possible with the Ched-OMatic method.

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OUR ASSOCIATION

REPORT OF COMMITTEE ON BUTTER, A.D.S.A.[1]

C. JENSEN, Chairman, E. W. BIRD, H. J. BUYENS, D. L. FOURT,
T. R. FREEMAN, AND S. R. HOOVER

Dairy departments in the provincial and state colleges and universities in Canada and the United States, and a research agency of the USDA, were surveyed relative to obtaining information concerning the butter research and testing activities presently under way. Returns from 52 respondents revealed that 14 research stations are presently engaged in research studies on butter or butterfat, and ten stations perform some type of testing service for butter-manufacturing plants. This report summarizes the information obtained relative to the butter research and testing activities at the various stations and also gives suggestions for further research.

COMMENTS

A continuous buttermaking machine suitable for processing plastic cream into butter has been designed and built at Alberta. Saskatchewan reported an increase in one-half cent per pound margin due to increased curd in unwashed as compared with washed butter. No difference in keeping quality was observed between the two types of butter. Similar observations relative to keeping quality were made at Iowa. This station also developed a procedure for the detection of butter adulteration with animal or vegetable fats, by the tocopherol method. It was reported that chromatography of fats on silica gel have not proven satisfactory, but high-vacuum distillation shows some promise, particularly on coconut oil. At Maryland, a method of characterizing butterfat by its butyric acid content has been developed and offered as a means of detecting butterfat adulteration. The Maryland workers are presently developing a colorimetric test for the butterfat content of dairy products. Also, they are working on the identification of flavor compounds in oxidized fat.

Investigation at Cornell indicated a correlation between tocopherol content of roughages fed to dairy cows and the tocopherol and carotenoid content of milk, as well as between the types of roughages fed and the resistance of milk fat to oxidative changes induced by copper and light.

Oregon reported that a softer and waxier butter can be produced during the winter by using a cream-temperature treatment after pasteurization. Investigators at Wisconsin also reported the development of procedures for improving body, texture, and spreadability. Also, they developed a device for measuring spreadability of butter. From the Utah station it was reported that an improvement in flavor, keeping quality, and spreadability was obtained with starter butter as compared with butter without culture.

Investigators at the Dairy and Meat Laboratories, Eastern Utilization Research and Development Division, Agricultural Research Service, USDA, found no improvement in the keeping quality of canned butter stored in an inert atmosphere. By intersterification of butteroil, an increased melting point of butterfat was obtained. In the Philadelphia Laboratory of the Division, a study of the highly unsaturated fatty acids of butter brought about the isolation of new isomeric forms of highly unsaturated fatty acids.

In reply to the question "What research on butter do you feel is needed?" 46 suggestions were offered and are tabulated as follows:

Suggested research projects	Number of suggestions
1. Body, texture, and spreadability	13
2. Physical structure of butter including *(a)* conventional and *(b)* continuous butter	2
3. Butter flavors including study of *(a)* the compounds that give butterfat the natural flavor, *(b)* uses of butter cultures for intensifying flavor, and *(c)* standardization of butter flavor	10
4. Keeping quality of different types of butter: Effect of *(a)* butter cultures; *(b)* oxidation defects; *(c)* study of antioxidants in butter and wrapping materials	6
5. Packaging of butter and butter spreads	3
6. Consumer preference studies including: Commercial acceptance of butter with different flavor intensities	2
7. Nutritive value of butterfat and butter, including: *(a)* composition of butterfat and its metabolism in the human body; *(b)* comparison of the nutritive value of butter with other fat foods	3

[1] Published according to the policy stated, *J. Dairy Sci.*, 40: 1369. 1957.

BUTTER RESEARCH

Institution		Nature of projects	Present status			Publications	
			Preliminary	In progress	Completed	No. published	No. ready for publication
Univ. Alberta, Canada	1.	Comparative study of conventional and continuous butter.			X	5	
	2.	Butterfat crystallization.		X			1
	3.	Setting of butter.		X		1	1
	4.	Gas content.			X	1	
	5.	Microscopy of the crystalline structure.		X		1	1
Univ. Manitoba, Canada	1.	Butter texture.		X		0	
	2.	Beta-carotene butter color.		X		0	
Univ. Saskatchewan, Canada	1.	Washed *vs.* unwashed butter.			X	1	
	2.	Effect of steel churn on quality.			X	1	
Univ. Arkansas	1.	Rancidity and bitter flavors in cream and butter.				2	
Univ. Idaho	1.	Consumer preference for different kinds of butter.		X		0	
Univ. Illinois	1.	Triglycerides in butterfat.		X		3	1
Iowa State College	1.	Detection of butter adulteration.		X		0	
	2.	Oxidation of fats and identification of compounds produced in oxidation.		X		2	4
	3.	Triglyceride structure.	X			0	1
	4.	Washed *vs.* unwashed butter granules.		X		0	
	5.	Butter manufacturing costs in whole milk creameries.				1	
	6.	Waste disposal in creameries.					1
Univ. Kentucky	1.	The water-soluble acid content of farm-separated cream.		X		0	
Univ. Maryland	1.	Detection of butterfat adulteration.			X	11	
	2.	Flavor of dairy products as affected by fat deterioration		X		0	
Univ. Nebraska	1.	Keeping quality of salted *vs.* unsalted butter.	X			0	
Cornell Univ. (N.Y.)	1.	Physico-chemical properties of milk fat.		X		70	2
Oklahoma A & M	1.	Influence of hydrogenation on the physical characteristics of butter.	X				1
	2.	Consumer preference for butter.		X		0	
South Dakota State College	1.	Consumer preference, demand and potential supply for butter.				1	
Utah State Univ.	1.	Comparative study of the use of starter in butter.	X			0	
Univ. Wisconsin	1.	Improvement of body, texture, and spreadability of butter.			X	2	
	2.	Whey cream butter.			X	2	
Dairy and Meat Lab., EURDD, ARS, USDA, Wash., D.C., and Philadelphia, Pa.	1.	Stability of butteroil as related to quality of butter.		X			
	2.	Canned butter.			X		
	3.	Interesterification of butteroil.		X			
	4.	Highly unsaturated fatty acids of butter.			X		1
	5.	Improved spreadability of butter.[a]		X			
	6.	Improved stability of butterfat.[b]		X			

[a] Project on contract with Ohio State University.
[b] Project on contract with Iowa State College.

BUTTER TESTING AND GRADING PROGRAMS

Institution	Cooperating agencies	Frequency of tests	Financed by	Creamery participation		Remarks
				Plants (%)	Butter (%)	
Univ. California	University	Monthly	Creamery	5–10	10	
Purdue	Creameries and Univ.	Monthly	Creamery	85	90	Considerable benefit past year. Creameries do not use analysis to best advantage.
Iowa State College	Creameries and I.S.C.	Quarterly, monthly, and weekly	Creameries on cost basis			Quarterly 14 plants. Fee $8 per sample. Creameries submit samples regularly on a once-a-week or a monthly cost basis. Occasional samples. Cost basis.
Montana State College	Coop. with State Dept. Agr. and USDA	Bimonthly	Federal funds		100	
North Dakota Agr. College	College N. Dak. Research Foundation and Creameries	Monthly	Grant by foundation. College	30	33	Improvement in butter quality, composition control, serum pH, extraneous material, and sanitation and skill in butter-making.
Ohio State Univ.	University	Monthly	Creamery	75	90	Improvement in accuracy of laboratory procedures, fat control, sanitation, and skill in butter-making.
Oklahoma A & M	Occasional samples submitted for analysis annually.					
Ontario Agricultural College	Creameries, College, and Government	Weekly		60	65	Weekly analysis for yeast and mold definitely raised quality of butter in general.
Univ. of Saskatchewan				25	35	Butter tested by provincial laboratory for pH and routine chemical analysis. 95% Saskatchewan butter 92 score or better.
Utah State Univ.		Irregular				Samples tested at short course.

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ABSTRACTS OF LITERATURE

W. O. Nelson, Abstract Editor

ANIMAL HEALTH

269. Effects of feeding fluorine, as sodium fluoride, to dairy cattle—a six-year study. G. W. NEWELL AND H. J. SCHMIDT, Stanford Research Inst., Menlo Park, Calif. Am. J. Vet. Research, **19**, 71: 363. 1958.

Twenty 2-yr.-old Holstein heifers were divided into five groups of four each and fed and managed in a normal, practical manner, except that four groups were given daily doses of NaF equal to 1.0, 1.5, 2.0, and 2.5 mg. of fluorine per kilogram of body weight, respectively. The control group ingested 0.15–0.3 mg. fluorine per kilogram. These amounts were equivalent to 7.3, 33.6, 44.7, 60.4, and 85.6 p.p.m. of the dry matter of the rations consumed. Records of milk production for six lactations are presented.

There was no indication that greater levels of fluorine within the limits tested affected milk yields. The control group averaged higher in fat test and fat yield than the treated groups, but this was believed due to the chance allotment of higher-testing heifers to this group. Conception rates of all groups were normal, with the control group being the lowest. Calves were normal in size and viability in all groups. Weight gains from 2 to 7 yr. were normal in all groups.

Urine fluorine concentrations varied widely within groups but, on the average, progressively higher fluorine feeding produced higher urine fluorine values. The normals averaged 2.6 p.p.m. Low levels of fluorine gave urine concentrations of 10–15 p.p.m., and levels high enough to cause damage to the cows averaged over 20 p.p.m. The most striking changes occurred in the incisor teeth, with excessive wear and mottling present in the third and fourth pairs of cows fed 2.0 mg/kg. The molar teeth of these cows showed no significant abnormalities. Other signs of toxicity noted were the "strained" attitude of most of the cows on the two highest levels, a rough coat and tight hide (especially during heavy lactation), and exostoses and lameness of cows on the highest level of fluorine.

All cows were slaughtered during their sixth lactation and bone and soft tissues examined for fluorine and histological abnormalities. With the exception of high fluorine contents, the only significant abnormalities observed were irregular bone structures in cows on the highest fluorine intake. These studies indicated that the borderline intake of fluorine for the production of toxicosis is about 2.0 to 2.5 mg. per kilogram body weight, or 60–85 p.p.m. in the dry feed. E. W. Swanson

270. Blood calcium in bovine ketosis. K. HALSE AND W. VELLE, Vet. Coll. Norway, Oslo, Norway. Am. J. Vet. Research, **19**, 72: 575. 1958.

The blood of 35 cows with varying degrees of ketosis was analyzed for acetone, sugar, calcium, and magnesium. In cows with blood acetone bodies above 45 to 50 mg. %, a statistically significant depression of blood calcium was observed. Magnesium also decreased at the highest acetone levels, but the correlation between it and acetone was not significant. Data from three cows were presented to show the relationship between reduced feed intake, hypocalcemia, and clinical signs of ketosis. Marked clinical signs were most evident when feed intake and blood calcium were low. If the blood calcium level and feed intake were normal, signs of ketosis were not evident, even though blood acetone was high. E. W. Swanson.

271. Further observations on silage as a possible etiological factor in bovine ketosis. J. H. ADLER, S. J. ROBERTS, AND J. A. DYE, Cornell Univ., Ithaca, N. Y. Am. J. Vet. Research, **19**, 71: 314. 1958.

Clinical observations indicated that on many New York farms ketosis in milk cows was associated with butyric acid silage. A method of assaying for acetone was developed and silage from 15 silos was analyzed. Farms which had the highest levels of acetone in the silage also had the highest incidence of ketosis in the herds. High acetone content was found in corn silage as well as grass silage, but highest levels were observed in grass silage, which contained excessive butyric acid. E. W. Swanson

272. Oxygen uptake in liver homogenates taken from normal and ketotic cows. F. SAUER, W. M. DICKSON, AND H. H. HOYT, Minn. Agr.

Expt. Sta., St. Paul. Am. J. Vet. Research, **19**, 72: 567. 1958.

Homogenates were made from 5–10 g. samples of liver tissue removed by incision from normal, lactating, and ketotic cows. Oxygen uptake of the liver homogenates was determined by the direct Warburg method on control samples as well as on those with added substrates, glucose, acetate, propionate, and acetoacetate. Glucose did not increase liver homogenate oxygen uptake, but acetate and acetoacetate did. Results with propionate were variable. The differences between normal and ketotic livers were quantitative more than qualitative. Oxygen uptake of ketotic liver was about one-half of normal with all substrates. An experiment with radioactive acetate showed that acetate utilization was not proportional to total respiration. Recovery of normal liver metabolism was followed in one cow for 5 wk. after the ketosis samples were removed. Normal appearance of the liver returned before normal metabolism.

E. W. Swanson

273. Post-parturient bovine uterine motility —normal and after experimentally produced retention of the fetal membranes. J. H. Venable and L. E. McDonald, Okla. Agr. Expt. Sta., Stillwater. Am. J. Vet. Research, **19**, 71: 308. 1958.

Puerperal uterine motility was determined in 19 normal cows by means of a 4-in. rubber balloon placed in the uterine horn as early as 2 hr. postpartum. Recordings made at intervals through 72 hr. showed that strong uterine contractions occurred initially at the rate of 14 per hour, then gradually reduced to less than one per hour at 42 hr. With time postpartum, the contractions were of less amplitude as well as less frequent. The copora lutea of six cows were removed at 215 to 242 days of gestation, to cause early parturition and retention of placenta. Placenta was retained by five of the six cows. All of these cows had higher than normal rates of uterine motility, averaging 19 strong contractions per hour. The rate of contractions remained high through three days of observations, but the amplitude decreased. The contractions were also undulating and tetanic, rather than simple and smooth, as in the normal cow.

E. W. Swanson

BOOK REVIEWS

274. Veterinary Handbook for Cattlemen. J. W. Bailey. Springer Publ. Co., Inc., New York, N. Y. 389 pp. $5. 1958.

The preface to the second edition of this book states that it differs from the first primarily in the addition of new material. The book is divided into 12 chapters. Chapter One is concerned with practical pointers for owners. Remaining chapters deal with infectious and noninfectious diseases of cows, bulls, and calves. The number of illustrations is limited but those included are instructive.

This book, as the title indicates, is intended for cattlemen. Although it is well written and nicely printed, it falls into the common error of nearly all such handbooks on diseases of animals; namely, confusing the owner of livestock by including a great deal of information that he simply can not use. To illustrate, the author himself states in the preface to the first edition that the correct dosage of medicines can often be determined only through careful examination by a trained observer, the veterinarian. Despite this injunction, he devotes many pages to treatment of disease and discusses at length various drugs and medicines the use of which taxes the knowledge and judgment even of the veterinarian.

Rapid progress has been made in recent years in human and veterinary medicine in the application of newer drugs, such as antibiotics and synthetic hormones, to the treatment of disease, but it is becoming all too evident that in many instances their use is not only expensive and ineffective but, in some cases, contraindicated and even dangerous.

The author has had wide experience as a practicing veterinarian. He has, therefore, not neglected to stress prevention. Cattlemen, especially dairymen, will find the book interesting and helpful if for no other reason than that it points out the importance of preventive measures in attempts to keep out and control many diseases that may affect some or all animals in a herd of cattle.

Jesse Sampson

CHEESE

275. Method for the curdling of milk. W. Meier and P. Zublin. U. S. Patent 2,848,400. 2 claims. August 19, 1958. Offic. Gaz. U. S. Patent Office, **733**, 3: 771. 1958.

Milk is prevented from sticking to the surface of stainless steel cheese vats by applying a negative electric potential with reference to the electric potential of the milk.

R. Whitaker

CONDENSED AND DRIED MILKS; BY-PRODUCTS

276. Process for preparing sterilized concentrated milk and product produced thereby. D. F. Wilcox. U. S. Patent 2,845,350. 7 claims. July 29, 1958. Offic. Gaz. U. S. Patent Office, **732**, 5: 1220. 1958.

High-temperature, short-time sterilized concentrated milk is stabilized with an extract of chondrus crispus at the rate of 0.0075 to 0.02% by weight.

R. Whitaker

277. L'emploi de la caséine comme stabilisant des peintures au latex. (Use of casein as stabilizer in latex paints). G. Génin. Lait, **38**, 276. 1958.

The author reports that casein or casein-based products can be used as stabilizers for the newer latex-based paints. The necessity for cooperation between the paint makers and casein manufacturers to assure the successful use of casein as a stabilizer is emphasized.

A. W. Rudnick, Jr.

DAIRY BACTERIOLOGY

278. Mise en évidence des streptocoques lactique inhibiteurs et stimulants dans le lait et les fromages, fréquence de ces streptocoques dans le lait cru et les fromages fermiers produits dans differentes régions de France. (Evidences of Streptococcus lactis inhibitors and stimulants in milk and cheese, frequency of streptococcus in raw milk and farm cheese produced in various areas of France). R. Chevalier, J. Fournaud, E. Lefebure, and G. Mocquot. Lait, **38**, 255. 1958.

To determine if *Streptococcus lactis* produces inhibiting or stimulating compounds, a medium was inoculated with a dilute suspension of *S. lactis* and the test organism, *Lactobacillus lactis*. The mixture was incubated in Petri dishes at a low temperature to permit growth of *S. lactis* and then at a higher temperature to encourage growth of the test organism. The plates were examined for growth of the test organism. A clear zone around *S. lactis* colonies was considered as evidence of inhibition. Increased growth at the colony indicated production of stimulatory substance.

In samples of raw milk and cheese from various areas of France it was found that 20 to 25% contained strains of *S. lactis* which produced inhibitory substances, but these were only about 2% of the total organisms studied. On the other hand, many of the samples contained *S. lactis* strains that stimulated the growth of the lactobacillus. A. W. Rudnick, Jr.

279. Utilisation du monolaurate de sorbitol comme agent émulsionnant des graisses dans les milieux destinés a la recherche des microorganismes lipolytiques (Use of sorbitol monolaureate as a fat emulsifier in media used in studies of lipolytic microorganisms). R. Alifax. Lait, **38**, 129. 1958.

Use of sorbitol monolaurate (Tween 20) made possible the preparation of media in which fat was in a fine and stable state of emulsion. The product was nontoxic to two types of lipolytic bacteria and one mold.

A. W. Rudnick, Jr.

DAIRY CHEMISTRY

280. Method for ascertaining the percentage of butter fat content of milk. E. M. Borg. U. S. Patent 2,844,067. 1 claim. July 22, 1958. Offic. Gaz., U. S. Patent Office, **732**, 4: 883. July, 1958.

One part of milk is diluted with two parts distilled water and the mixture emulsified. A beam of light is directed through a color filter, then through a layer of the emulsion to a photoelectric cell. A meter measuring the stimulation of the cell is calibrated to read butter fat percentage directly. R. Whitaker

281. Dosage simultané du fer et du cuivre dans le lait et les produits laitiers (Simultaneous determination of iron and copper in milk and dairy products). R. Alifax and M. Bejambes, Station Centrale de Microbiologe et Recherches laitières, Jouy-en-Josas. Lait, **38**, 146. 1958.

The method of Ventura and White for simultaneous determination of iron and copper in blood has been adopted for use in dairy products. The method is based on the fact that iron gives a yellow and copper a red color reaction with 2-2 dipyridyl. The two fractions are separated by the extraction of copper in an ethyl ether–amyl alcohol mixture. The separated fractions are examined spectrophometrically with reference to a standard curve.

A. W. Rudnick, Jr.

PHYSIOLOGY AND ENDOCRINOLOGY

282. Elimination of phenothiazine by lactating dairy cows. T. Richardson and A. C. Todd, Wis. Agr. Expt. Sta., Madison. Am. J. Vet. Research, **19**, 72: 610. 1958.

Phenothiazine (100 g.) was administered to five cows in starch-pressed boluses and to two cows as a capsule of micronized phenothiazine. Total collections of feces, urine, and milk were made until excretion of phenothiazine ceased. Much of the drug from the boluses passed through the cow unchanged; whereas, the micronized drug was more completely absorbed. From the boluses, 62.4% appeared in the feces, 19.0% in the urine, and 0.07% in the milk. From the capsules (micronized), 22.7% appeared in the feces, 54.3% in the urine, and 0.29% in the milk. Phenothiazine appeared in the first milking after dosing, as early as 5.5 hr., and was detectable for 71 hr. after boluses and 86.5 hr. after capsules. These elimination times corresponded to 79.0 and 71.5 hr. in feces, and 112 and 132.5 hr. in urine for boluses and capsules, respectively. As little as 2 p.p.m. could be detected in milk by colorimetric methods. Cows given 2-g. doses daily failed to show phenothiazine in their milk, and there was no indication that this dose level caused any abnormalities of milk. The cows given 100-g. doses secreted milk which readily developed a pink color while phenothiazine was being excreted.

E. W. Swanson

283. Effects of diethylstilbestrol feeding on the bovine reproductive tract. H. W. Reuber, Iowa State Coll., Ames. Am. J. Vet. Research, **19**, 72: 585. 1958.

Three lots of six yearling heifers each were fed 0, 10, and 20 mg. of stilbestrol daily in a fattening ration for 148 days. No genital abnormalities were noted and no differences between lots in frequency or duration of estrus. After 148 days, half of the heifers were slaughtered and the others placed with a bull and stilbestrol feeding stopped. All heifers conceived and fetuses were normal at slaughter 91 days after stopping stilbestrol. Feces from the stilbestrol-fed heifers averaged 1.06 μg. stilbestrol per gram. The 10- and 20-mg. groups excreted 43.8 and 42.5%, respectively, of ingested stilbestrol in the feces. Another lot of 24 heifers was observed in three groups as above on a growing, rather than fattening, diet. Again, no genital abnormalities or pronounced abnormalities of behavior were noted. However, these stilbestrol-treated heifers were noted in estrus more often and for longer periods than were the control heifers. Three groups of seven each of 7-mo.-old bulls also were used to compare feeding 0, 10, and 20 mg. of stilbestrol daily. No effects attributable to stilbestrol treatment were observed in feed lot behavior, testicular size, or in fertility as measured by semen quality. E. W. Swanson

SANITATION AND CLEANSING

284. Apparatus for cleaning pipelines. Z. O. St. Palley, U. S. Patent 2,845,935. 5 claims. August 5, 1958. Offic. Gaz., U. S. Patent Office, 733, 1 : 93. 1958.

This apparatus is an arrangement of two tanks for flushing cleaning solutions through a pipeline. The solution is forced from one tank to the other by means of compressed air. An electrical system makes the operation automatic. R. Whitaker

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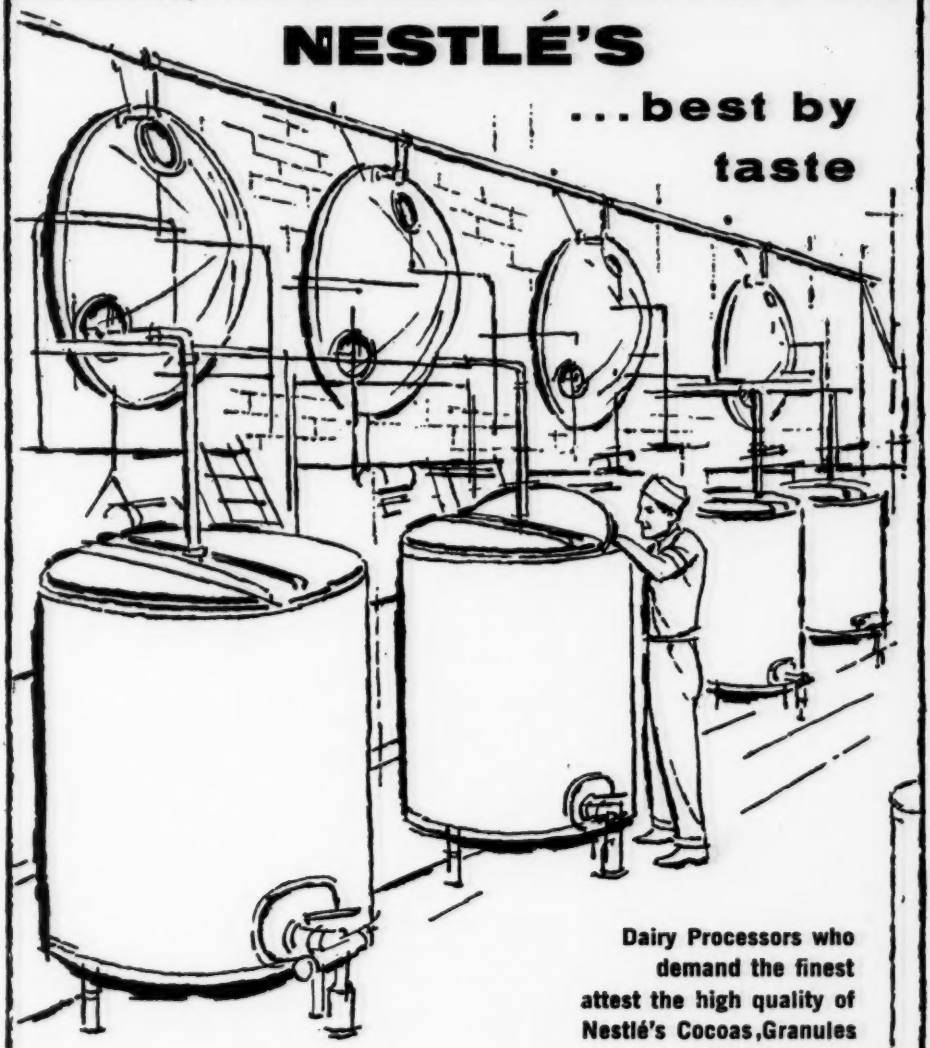

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